# Neuromuscular Synaptogenesis: Role of a Balance Between Tyrosine Kinases and Phosphatases

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# Neuromuscular Synaptogenesis:

# Role of a Balance Between

# Tyrosine Kinases and Phosphatases

submitted in fulfilment of the requirements of the
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# Zusammenfassung

Die neuromuskuläre Synapse (NMS) ist die zugänglichste und best-charakterisierte Synapse and dient seit Jahrzehnten als Modell für Synapsenbildung. Molekulare Abläufe während ihrer Entwicklung sind anfänglich unabhängig vom Nerv, aber später dominieren Prozesse, die durch die Ankunft des Nervs am Muskel angetrieben werden. Das vom Nerv ausgeschüttete Protein Agrin aktiviert die Muskel-spezifische Kinase MuSK und fördert deren Tyrosin-Phosphorylierung. Signalisierungsprozesse stromabwärts von MuSK führen zur Aggregation der Acetylcholin-Rezeptoren (AChR), wozu das Ankerprotein Rapsyn nötig ist. AChRen werden durch Agrin phosphoryliert; dieser Vorgang resultiert nicht direkt aus der Kinase-Aktivität von MuSK sondern aus anderen Tyrosinkinasen. Das Ausmass der Phosphorylierung von MuSK und AChR wird durch Tyrosinphosphatasen kontrolliert.

Src-Familien Kinasen (SFKen) wurden als Kinasen identifiziert, die an MuSK und auch AChRen binden. Abelson-Kinase (Abl)-Familienmitglieder waren ebenfalls nötig zur Aggregation der AChRen, und MuSK und Abl-Kinasen phosphorylierten sich gegenseitig. Deshalb nimmt man an, dass SFKen und Abl-Kinasen die Kinase-Aktivität liefern, die stromabwärts für die Verbreitung des Agrin-Signals notwendig ist. In dieser Dissertation habe ich ein zeitliches Muster der Aktivität dieser zwei Kinase-Familien entdeckt, das die Phosphorylierung von AChR und MuSK bewirkt. Ich fand heraus, dass nach Agrin-Zugabe zu Muskelzellen SFKen für frühe (5 Min.) Phosphorylierung von MuSK und AChR zuständig sind, während Abl-Kinasen späte (nach 40 Min.) Phosporylierung bewirken. SFK-Aktivität während der Agrin-Zugabe ist nicht notwendig für Akkumulation des AChRs, aber es braucht sie zur Stabilisierung der AChR-Clusters später, nach Wegnahme von Agrin. Zudem konnte ich zeigen, dass ein einzelner kurzer Puls von Agrin ausreichend ist, um in Muskelzellen effiziente Phosphorylierungen und AChR-Aggregation zu erreichen, solange eine Schwellen-Aktivität von MuSK erzielt wird. Diese Daten zeigen, dass stromabwärts gelegene Tyrosinkinasen die Aktivität der Agrin-Signalisierungsprozesse hochhalten.

Phosphatasen ihrerseits sind wichtig für die Agrin-induzierte AChR-Aggregation und die Stabilität dieser Clusters nach Agrin-Wegnahme: Ich fand heraus, dass Inhibition von Phosphatasen durch Pervandat die Bildung und Stabilisierung der Clusters stört. *src*$^{-/-}$;*fyn*$^{-/-}$ Muskelzellen und Wildtyp-Zellen, deren SFKen pharmakologisch blockiert wurden, zeigten unstabile AChR-Clusters nach Wegnahme von Agrin. Jedoch konnte ich zeigen, dass gleichzeitige Inhibition der Phosphatasen die Clusters wieder stabilisiert. Demnach ist ein präzises Phosphorylierungs-Gleichgewicht für die Stabilität der AChR-Aggregate nötig nach Wegnahme von Agrin aus dem Zellkulturmedium, und das Gleichgewicht wird durch SFKen und Phosphatasen eingestellt. SHP-2 ist eine gute Kandidatin, da diese Phosphatase in Muskeln reichlich vorhanden und an NMSen konzentriert ist; ich konnte zeigen, dass SHP-2 zunehmend an MuSK bindet nach Behandlung mit Agrin. Ein „Knockdown" der Proteinmenge von SHP-2 durch Vektor-getriebene shRNA (RNA Interferenz) führte einerseits zu einer Reduktion der Menge an AChR-Clusters, an unbehandelten wie Agrin-behandelten Zellen; andererseits war das Potential der Agrin-Signalisierung verstärkt. Ein wichtiger Aspekt des SHP-2-Knockdowns war die resultierende Unstabilität der durch Agrin induzierten AChR-Clusters. Zudem war die Proteinmenge von SHP-2 in *src*$^{-/-}$;*fyn*$^{-/-}$ Muskelzellen erhöht; dies lässt vermuten, dass in diesen Mutanten ein hohe Phosphatasenaktivität zusammen mit weniger Kinaseaktivität (da Src und Fyn fehlen) zu einem Ungleichgewicht an Phosphorylierungen führt, was AChR-Clusters unstabil werden lässt.

Zusammenfassend präsentiere ich in dieser Dissertation Ergebnisse, die die Kontrolle durch Tyrosinkinasen und Phosphatasen in molekularen Signalisierungsprozessen an der NMS belegen. Kinasen sind wichtig um Phosphorylierungen voranzutreiben und Agrin-Signalprozesse zu verstärken, während der Bildung von AChR-Aggregaten und zu ihrer Stabilisierung. Phosphatasen wie SHP-2 geben Gegensteuer und sind ebenfalls nötig zur Aufrechterhaltung der Clusters. Deshalb scheint eine genaue Balance zwischen Kinasen und Phosphatasen die Stabilität molekularer Signalvorgänge an der sich entwickelnden NMS, und damit die Stabilisierung dieser Synapse an sich, zu begünstigen.

# Summary

The neuromuscular junction (NMJ) is the most accessible and best-studied synapse, serving as a model for investigating synaptogenesis for decades. Molecular events occurring during the formation of this synapse are initially driven partly by nerve-independent mechanisms, but later pathways stimulated by the arrival of the nerve at the muscle membrane take over. A nerve-released factor called agrin activates the muscle-specific receptor tyrosine kinase (MuSK) causing its tyrosine phosphorylation. MuSK downstream signalling leads to the clustering of acetylcholine receptors (AChRs), which requires the presence of the anchor protein rapsyn. The AChR is also phosphorylated by agrin. This phosphorylation is however not a direct result of MuSK kinase activity, but requires other non-receptor protein tyrosine kinases (PTKs). The level of phosphorylation of MuSK and the AChR is controlled in turn by protein tyrosine phosphatases (PTPs).

Src-family kinases (SFKs) were identified as one family of PTKs that associate with both MuSK and AChRs. Abelson kinase (Abl) family members were also shown to be required for efficient AChR cluster formation, and MuSK and Abl exhibited reciprocal tyrosine phosphorylation. Therefore, SFKs and Abl kinases are thought to provide the downstream kinase activity required for the propagation of the agrin signal. In this thesis I have identified a distinct temporal pattern of activity of these two PTK families which results in the phosphorylation of MuSK and the AChR. I found that SFKs are responsible for early (5 min) phosphorylation of MuSK and AChRs, whereas Abl kinases control later MuSK and AChR phosphorylation (after 40 min) following agrin addition to myotubes. SFK activity during the agrin induction period is not required for AChR clustering, but is required for the stability of these agrin-induced clusters later, following the withdrawal of agrin. Furthermore, a single brief agrin pulse is sufficient to induce efficient phosphorylation and maximal clustering of AChRs as long as a threshold level of MuSK activation is achieved. These data imply that downstream tyrosine kinases maintain the agrin signalling pathway.

PTPs, in turn, are important for agrin-induced AChR clustering and stability of these clusters after agrin removal: I found that blocking PTPs with the inhibitor pervanadate interferes with cluster formation and stability. *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes as well as SFK inhibitor-treated wild-type myotubes display unstable AChR clusters upon agrin withdrawal. However, my results show that concomitant PTP inhibition upon agrin withdrawal rescues AChR cluster stability. Thus a fine phosphorylation balance is required for AChR cluster stability following withdrawal of agrin from myotube cultures, and is controlled by an equilibrium between SFK and PTP activities. SHP-2 is a good candidate PTP since it is abundant in muscle at the NMJ; it colocalises with AChR clusters *in vivo*, and I found that it increasingly associates with phosphorylated MuSK upon agrin stimulation in myotubes. Knocking down SHP-2 in myotubes using vector-driven shRNA resulted on one hand in a reduction in AChR clusters in untreated and agrin-treated myotubes; on the other hand, SHP-2 knockdown caused an increase in the agrin induction potential for AChR clustering. A significant effect of knockdown of SHP-2 was the resulting instability of agrin-induced AChR clusters following the withdrawal of agrin from myotubes in culture. I also found an increased protein level of SHP-2 in *src*$^{-/-}$;*fyn*$^{-/-}$ compared to wild-type myotubes; this suggests that in mutant myotubes an increased overall phosphatase activity combined with a reduced kinase activity due to absence of Src and Fyn may lead to an imbalance in phosphorylation levels, causing instability of AChR clusters induced by agrin.

In summary, in this thesis I present findings demonstrating the control by tyrosine kinases and phosphatases of molecular signalling events driven by agrin in the postsynaptic density of the NMJ. Tyrosine kinases are essential to drive phosphorylation events and amplify the agrin-MuSK signal during postsynaptic clustering of AChRs, and for maintenance of stability of these clusters. Tyrosine phosphatases, such as SHP-2, counterbalance this activity, and are also critical for AChR cluster stabilisation. Therefore a fine balance of kinase versus phosphatase activity seems to be required to maintain stable molecular signalling, and finally a stable synapse, at the developing NMJ.

# Table of Abbreviations

| | |
|---|---|
| Abl | Abelson tyrosine kinase |
| ACh | acetylcholine |
| AChE | acetylcholinesterase |
| AChR | acetylcholine receptor |
| α-BTX | α-bungarotoxin |
| AMPA | α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| APC | Adenomatous Polyposis Coli |
| BDNF | brain-derived neurotrophic |
| CNS | central nervous system |
| D/UGC | dystrophin/utrophin glycoprotein complex |
| DB | dystrobrevin |
| DG | dystroglycan |
| DMEM | Dulbecco's modified Eagle’s medium |
| DNA | deoxyribonucleic acid |
| Dvl | dishevelled |
| ECM | extracellular matrix |
| EGF | epithelial growth factor |
| FGF | fibroblast growth factor |
| GABA | γ-amino butyric acid |
| GGT | geranylgeranyltransferase I |
| GTP | guanosine triphosphate |
| HSPG | heparan sulphate proteoglycan |
| Ig | immunoglobulin |
| LTD | long-term depression |
| LTP | long-term potentiation |
| MASC | myotube-associated specificity component |
| MG | myasthenia gravis |
| MuSK | muscle-specific receptor tyrosine kinase |
| NCAM | neural cell adhesion molecule |
| NGF | nerve growth factor |

| | |
|---|---|
| NMDA | *N*-methyl-D-aspartate |
| NMJ | neuromuscular junction |
| NT-4 | neurotrophin-4 |
| PAK | p21-activated kinase |
| PNS | peripheral nervous system |
| PP2 | 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine |
| PSC | perisynaptic Schwann cell |
| PTB | phosphotyrosine-binding domain |
| PTK | protein tyrosine kinase |
| PTP | protein tyrosine phosphatase |
| pY | phosphotyrosine |
| rapsyn | receptor-associated protein at the synapse |
| RATL | rapsyn-associated transmembrane linker |
| RNA | ribonucleic acid |
| RNAi | RNA interference |
| SDS | sodium dodecyl sulphate |
| SEM | standard error of the mean |
| SFK | Src-family kinase |
| SH2 | Src homology 2 domain |
| SH3 | Src homology 3 domain |
| SHP-2 | Src homology 2 domain-containing phosphatase 2 |
| shRNA | short-hairpin RNAi |
| siRNA | small-interfering RNA |
| Src | v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homologue (avian) |

# Chapter 1

## *Introduction*

## 1.1 The synapse

In 1856, in Paris, Claude Bernard made the observation that the poison *curare*, used on South American arrow tips, caused the paralysis of muscles of frogs which were injected with the poison. He deduced that the action was not on the muscle itself, but on the nerve, pointing to the junction between the nerve and muscle as a possible site of action. In 1872, German Willy Kühne published a series of observations from several species, of nerve endings juxtaposed to a specialisation on the underlying muscle, and coined the term neuromuscular junction (NMJ). His work was the start of an era for the NMJ to be used as a model for the study of the contact between excitable cells, and for transmission between these two cells. However, the inability to visualise these contacts in great detail, due to limitations of visualising techniques in those days, led Santiago Ramon y Cajal and Camillo Golgi to develop the reticular theory of nervous organisation. Golgi developed staining methods for visualising individual neurones, and Ramon y Cajal applied the Golgi stain to map and describe the cellular architecture of the cerebellum. He noted that "the propagation of nervous action is made by contacts at the level of certain apparatuses or dispositions of engagement." Cajal's observations came at the advent of the cell theory, and the terminology was set for nerve cells to be called neurones, short processes emerging from the cell body to be called dendrites, and the long fibres previously called the axis cylinder became known as axons. It was only until the British neurophysiologist Charles Sherrington (Figure 1.1), working on the reflex arc in the spinal cord, in revising the nervous system section of the standard *Textbook of Physiology*, proposed "So far as our present knowledge goes, we are led to think that the tip of a twig of the arborescence is not continuous with but merely in contact with the substance of the dendrite or cell body on which it impinges. Such a special connection of one nerve cell with another might be called a synapse." The term *synapse* was finally in existence. Further developments brought about by neurophysiologists, and the dawn of molecular biology, resulted in huge leaps in our understanding of how the synapse works. The NMJ always served as the easiest and most accessible synapse to help us understand how these specialised cell to cell contacts work, and of course, insights

into NMJ functioning allowed to understand the connection between the brain and spinal cord and muscle contraction (Shepherd and Erulkar, 1997).

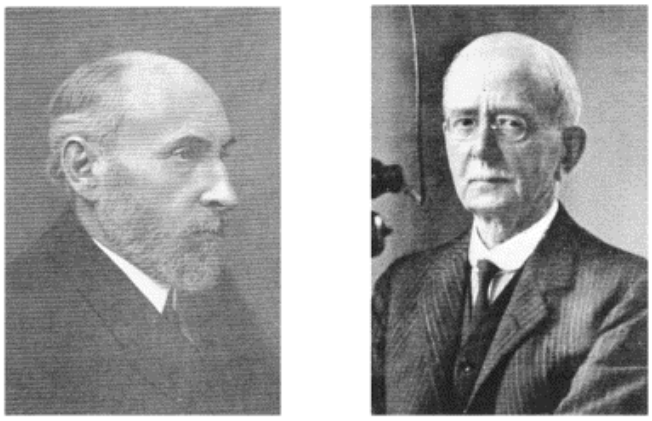

**Figure 1.1.** The fathers of the synapse. Santiago Ramon y Cajal (1852–1934) (left) used the Golgi stain to study the cellular architecture of the cerebellum. The British neurophysiologist Charles Scott Sherrington (1858–1952) (right) coined the term *synapse* (adapted and modified from Bennett, 1999).

## 1.2 The neuromuscular junction as a model synapse

The vertebrate skeletal NMJ is the specialised synapse formed between motor neurones and muscle fibres. It has served as a model for the study of the function and development of synapses for over fifty years (Sanes and Lichtman, 1999). The practicality of its accessibility and size has made it the synapse of which we know most about in terms of function and formation. There is however still lots left to discover, especially with regards to the molecular aspects of the NMJ.

The presynaptic nerve terminal approaches the muscle during development, and on making contact with the muscle membrane, it forms specialisations adapted for neurotransmitter release. These include high concentrations of synaptic vesicles, containing the neurotransmitter acetylcholine (ACh), ready for release at active zones. Large accumulations of mitochondria provide the energy for efficient exocytosis to take place in order to achieve a fast and efficient transmission of the signal across to the muscle. The nerve terminal is in turn capped by a perisynaptic Schwann cell (PSC) (Figure 1.2). PSCs are not thought to play a role during the nerve-muscle contact, but rather to lead synaptic growth and to play a role in the extension and

maturation of developing NMJs (Herrera and Ko, 2000). Specialisations on the postsynaptic muscle membrane are numerous and elaborate. They begin with structural changes involving a depression of the membrane below the nerve terminal into a gutter. Further invaginations of the membrane into folds, about 1 μm deep, allows for an increase in the total surface area of the muscle exposed to the presynaptic nerve terminal, increasing the efficiency of neurotransmission. The invaginations fall right beneath active zones of the nerve terminal. Acetylcholine receptors (AChRs) cluster on the crests of the junctional folds, whilst sodium channels and neural cell adhesion molecule NCAM accumulate in the troughs, also features that improve the efficiency of synaptic transmission.

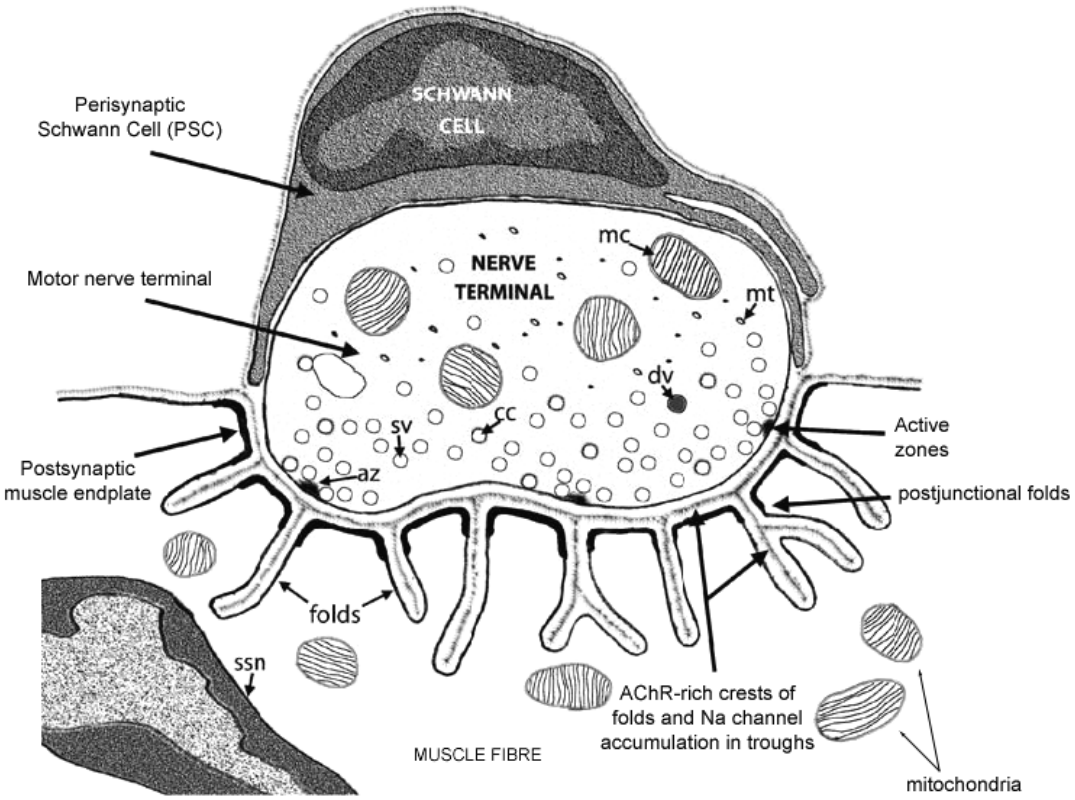


**Figure 1.2.** The neuromuscular junction is the specialised synapse formed between a motor neurone and a muscle fibre, and supported by a perisynaptic Schwann cell which caps the motor neurone. The nerve terminal occupies a shallow depression in the muscle fibre. The nerve terminal forms active zones (az) which directly appose junctional folds in the postsynaptic membrane. The illustration represents observations typically seen in thin sections of adult mammalian NMJs, in the transmission electron microscope. Abbreviations are: mc, mitochondria; mt, microtubules; dv, dense core vesicle; cc, clathrin-coated vesicle; sv, synaptic vesicle; ssn, sub-synaptic myonuclei (adapted and modified from Patton, 2003).

Separating the neurone and the muscle membrane in the synaptic cleft is a basal lamina, consisting of typical molecules such as collagen IV, laminin, entactin, and heparan sulfate proteoglycans (Sanes and Lichtman, 1999). Several of these molecules play very important roles during the development of the synapse, and later on in adulthood, in maintaining a stable synapse.

## 1.3 The postsynaptic density

### *1.3.1 The model*

Key to forming a fully functional synapse is the accumulation of a very high density of AChRs (10-20,000 per $\mu m^2$) at the crests of the junctional folds (in contrast to 1000-fold less extrasynaptically). These high densities allow for a rapid and reliable synaptic transmission (Sanes and Lichtman, 1999; Strochlic et al., 2005). This accumulation of neurotransmitter receptors is key, and is also paralleled in the central nervous system (CNS), during the development of glutamatergic synapses (Husi et al., 2000). During synaptogenesis, important events lead to the development of the neuromuscular synapse. First, the arrival of the nerve triggers the clustering of AChRs to the region of the muscle right beneath the nerve terminal. Pre-existing AChR clusters are stabilised if overlaid by nerve terminals but eliminated if present at a distance to the nerve (Kummer et al., 2006). Secondly, there is an accumulation of synaptic myonuclei beneath the synapse, and an upregulation of the transcription of synaptic genes. Thirdly, there is a shutdown of synaptic gene transcription in extrasynaptic nuclei (Huh and Fuhrer, 2002; Kummer et al., 2006; Sanes and Lichtman, 1999; Sanes and Lichtman, 2001).

### *1.3.2 The key players*

A neurally-released signal called agrin has been shown to be one of the key players during NMJ development, owing to its synaptogenic properties (McMahan, 1990). Agrin accumulates in the basal lamina, and stimulates the activation of the

muscle-specific receptor tyrosine kinases (MuSK) on the postsynaptic muscle membrane (Figure 1.3; DeChiara et al., 1996). MuSK phosphorylation leads to recruitment of signalling molecules and downstream signalling events which result in the clustering of AChRs at very high densities in the muscle membrane. The accumulation of AChRs has been shown to require the presence of several molecules. Acting downstream of MuSK is the protein rapsyn (receptor-associated protein at the synapse), shown to be required for clustering of AChRs to occur (Gautam et al., 1995). But what makes agrin special in its synaptogenic properties, and how are these downstream signals transmitted and executed by MuSK and rapsyn?

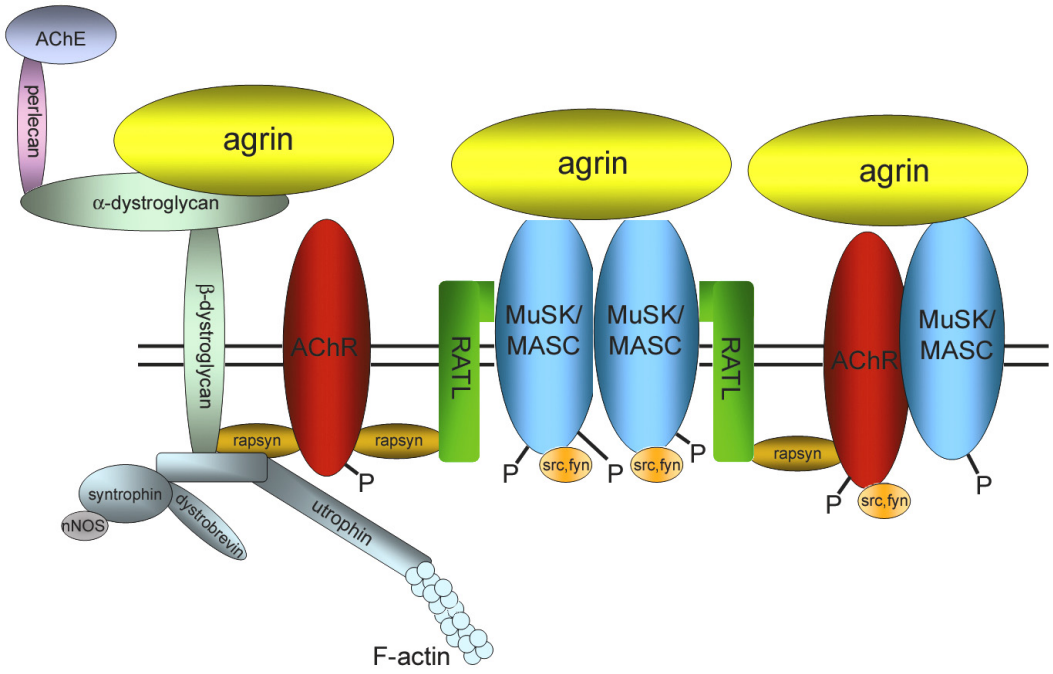


**Figure 1.3.** The postsynaptic density of the NMJ. Major postsynaptic proteins and their interactions are depicted in this scheme. For simplicity, not all protein isoforms are shown. Abbreviations: p, tyrosine phosphorylation; MuSK, muscle-specific kinase; MASC, myotube-associated specificity component; RATL, rapsyn-associated transmembrane linker; AChE, acetylcholinesterase (adapted and modified from Willmann and Fuhrer, 2002).

### *1.3.3 All orchestrated by agrin*

The discovery by McMahan and colleagues in the early 1980s that the extracellular matrix (ECM) molecules of the basal lamina of the synaptic cleft had indeed

synaptogenic properties, led them to the discovery of agrin. Agrin was isolated from the electric organ of *Torpedo californica*. It was discovered based on its ability to induce AChR clusters (Godfrey et al., 1984; Nitkin et al., 1983). Agrin has a predicted molecular weight of ~225 kDa, with N- and O-linked glycosylation increasing this to an apparent ~600 kDa. It is a heparan sulphate proteoglycan (HSPG) having multiple domains (Ruegg et al., 1992; Rupp et al., 1991; Tsim et al., 1992). The structure of agrin is summarised in Figure 1.4. It consists of nine cysteine-rich follistatin-like domains (F) in its N-terminal half, with two laminin EGF-like regions (L) between the eighth and ninth F domain. Two serine/threonine-rich regions (ST) in the central portion are followed by four EGF-like repeats (E) and three laminin G-like domains (LG) on its C-terminus. Agrin is glycosylated at several sites along its whole length (Bezakova and Ruegg, 2003; Luo et al., 2003a).

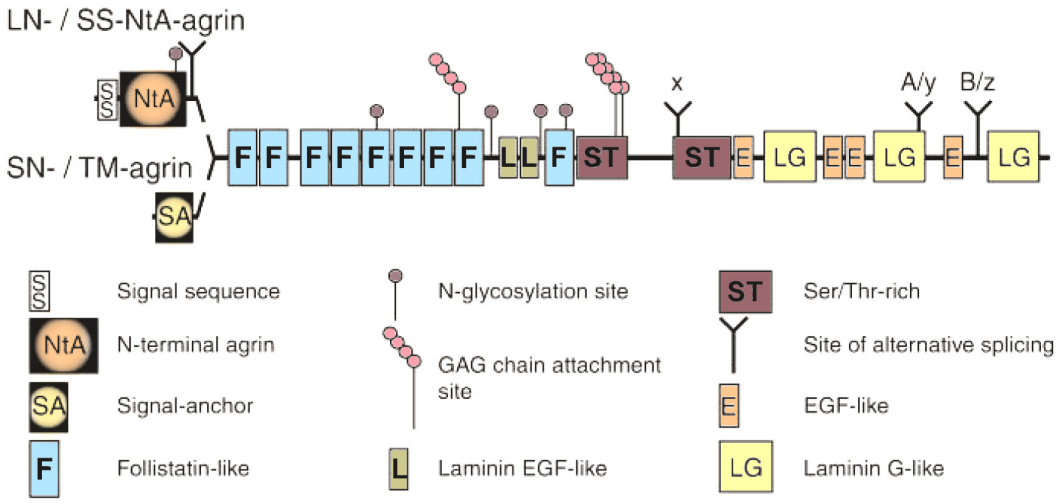


**Figure 1.4.** Schematic representation of agrin. Agrin is a heparan sulphate proteoglycan expressed by both the motor neurone and the muscle. However the splice variant produced by the neurone, containing the B/$z^+$ inserts, is 1000-fold more active in clustering AChRs (adapted and modified from Willmann and Fuhrer, 2002).

Agrin expression is not restricted to neurones. It is also detected in skeletal muscle, lungs, kidneys, glia and Schwann cells (Ruegg et al., 1992). Alternative RNA splicing of the agrin gene generates several splice isoforms of the protein, possessing distinct activities and distributions. The inclusion of inserts at the C-terminus of agrin ($B^+$ in birds; $z^+$ in mammals) greatly potentiates agrin's ability to induce AChR clustering.

The $z^+$ agrin isoform expressed by motor neurones is about 1000-fold more active than muscle agrin, which lacks this insert (Ferns et al., 1992; Ferns et al., 1993; Ruegg et al., 1992). N-terminal splicing of the agrin gene results in a long isoform (LN) or a short isoform (SN) of agrin, with LN isoform being required for NMJ development (Burgess et al., 2000; Luo et al., 2003a; Sanes and Lichtman, 1999), and SN isoform, which carries a transmembrane signal-anchor domain (SA) being most prominent in the brain (Burgess et al., 2002; Neumann et al., 2001).

Agrin synthesised by the nerve, and released into the ECM at the synapse, accumulates in the synaptic basal lamina. The absence of agrin in *agrin*$^{-/-}$ mice leads to the failure of NMJs to form and to differentiate, with disturbed intramuscular nerve branching, no nerve-associated clusters of AChRs or other postsynaptic proteins, and disturbed presynaptic differentiation (Gautam et al., 1996). Further evidence of agrin's critical role as the main synapse-inducing factor came when it was extra-synaptically injected into muscles; this led to local accumulation of a full postsynaptic apparatus (Cohen et al., 1997; Meier et al., 1997).

Agrin is capable of interacting with several muscle proteins present in the postsynaptic membrane. The N-terminal region of agrin interacts with laminins, HB-GAM, FGF-2, as well as NCAM. Its C-terminus associates with dystroglycan, HSPGs and integrin (Luo et al., 2003a; Sanes and Lichtman, 1999). *In vivo*, the combination of N-terminal and C-terminal regions is essential for the formation and maintenance of the postsynaptic apparatus (Meier et al., 1998). However, the actual AChR clustering activity of agrin is present in its C-terminal region. Maximal clustering is still achieved by a small 21 kDa region consisting of the LG3 domain containing the B/z inserts. This leads to the conclusion that agrin must interact with its functional receptor via this LG3-B/$z^+$ domain (Bezakova and Ruegg, 2003; Gesemann et al., 1996).

## *1.3.4 Agrin signalling: MuSK and the rest of the team*

### 1.3.4.1 MuSK

Agrin induces rapid phosphorylation of the type I receptor-like tyrosine kinase MuSK on the postsynaptic muscle membrane. MuSK was discovered due to its abundance in the synapse-rich electric organs of *Torpedo californica* (Jennings et al., 1993). MuSK is a transmembrane protein possessing four extracellular immunoglobulin (Ig)-like domains that are conserved across species (Figure 1.5). The first Ig-like domain is responsive to agrin (Zhou et al., 1999), whilst the fourth is required for clustering of MuSK with rapsyn via a putative transmembrane linker protein termed RATL (rapsyn-associated transmembrane linker) (Apel et al., 1995). Intracellularly MuSK possesses a 50-amino acid juxtamembrane region (containing an NPXY motif), a kinase domain, and a 13-amino acid C-terminal region (Valenzuela et al., 1995).

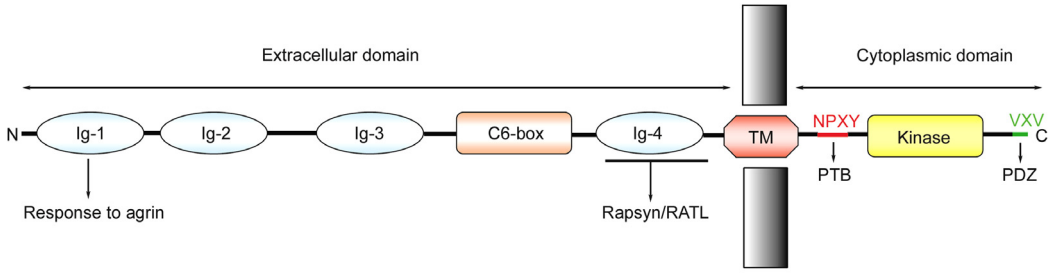


**Figure 1.5.** The muscle-specific kinase (MuSK) forms part of the agrin receptor. This schematic representation shows the important domains of MuSK and their interactions. The C-terminal juxtamembrane NPXY motif and the kinase domain are required for AChR clustering by agrin. Abbreviations: Ig, immunoglobulin domain; TM, transmembrane domain; NPXY, PTB-domain binding motif ; VXV, PDZ-binding motif (adapted and modified from Strochlic et al., 2005).

MuSK phosphorylation is brought about through its dimerisation. In stimulating MuSK, agrin does not directly bind to it. This led to the proposal of a co-receptor, termed MASC (myotube-associated specificity component), to associate with MuSK thereby together forming the functional receptor for agrin. The identity of MASC has so far remained elusive, and could also simply constitute a post-translational modification of MuSK itself (such as glycosylation) (Glass et al., 1996). MuSK

activation rapidly leads to clustering of AChRs. The absolute requirement for MuSK for postsynaptic development is seen in *musk*$^{-/-}$ mice which lack differentiation of the NMJs, with complete absence of AChR clusters, and other postsynaptic proteins (DeChiara et al., 1996). Agrin fails to induce AChR clustering in *musk*$^{-/-}$ myotubes in culture, but the phenotype is rescued upon transfection of wild-type MuSK. Knockouts of agrin and MuSK have very similar phenotypes, although one major difference is that in *musk*$^{-/-}$ mice AChRs fail to cluster at all, whilst in *agrin*$^{-/-}$ mice there are many transient AChR clusters in development and still a few AChR clusters at nerve-muscle contacts at birth (Glass and Yancopoulos, 1997; Lin et al., 2001; Yang et al., 2001).

The phosphorylation of MuSK is a crucial event in propagating downstream signalling leading to the clustering of AChRs. Agrin causes phosphorylation of MuSK on six of its tyrosine residues (Watty et al., 2000). Through mutational analysis two tyrosines have been shown to be absolutely required for agrin-induced AChR clustering. One tyrosine residue lies in the activation loop of the MuSK kinase domain, and the second lies in the NPXY motif in the juxtamembrane region (Y553) (Herbst et al., 2002; Herbst and Burden, 2000; Zhou et al., 1999). This shows the importance of the kinase activity of MuSK in response to agrin. The NPXY motif in the juxtamembrane region is a potential binding site for phosphotyrosine-binding (PTB) domain binding proteins, which could be important for recruitment of downstream signalling partners and adaptor proteins. MuSK-TrkA chimeras have been created expressing the MuSK juxtamembrane region on a TrkA kinase domain. When this chimeric receptor was expressed in MuSK mutant mice, pre- and postsynaptic differentiation was restored indicating the requirement of this juxtamembrane tyrosine for many aspects of MuSK signalling (Herbst et al., 2002; Herbst and Burden, 2000).

Agrin's synaptogenic properties manifest themselves via MuSK activation and downstream signalling, the end result of which is the clustering of AChRs and associated partners in the postsynaptic density. AChRs also require the presence of other proteins acting downstream of MuSK, for clustering to take place in response to agrin signalling. A key player among these proteins is rapsyn.

### 1.3.4.2 Rapsyn

Rapsyn is a 43 kDa scaffolding protein which is necessary for agrin-induced AChR clustering at the NMJ. It was also discovered in *Torpedo* synaptic membranes, closely associating with AChRs (Burden et al., 1983). It contains an N-terminal myristoylation sequence important in membrane targeting of rapsyn (Figure 1.6). This is followed by eight tetratricopeptide repeats (TPR) which result in rapsyn's self-association in heterologous cells (Ramarao et al., 2001). An α-helical amphipathic sequence overlaps with the eighth TPR. This is predicted to be a coiled-coil structure required for binding and clustering AChRs. At the C-terminal portion rapsyn possesses a cysteine-rich zinc finger (RING-H2) domain thought to be necessary to stabilise AChRs, and in linking rapsyn to the cytoskeleton (Banks et al., 2003). Rapsyn binds directly and clusters with AChRs in a 1:1 stoichiometry at adult NMJs (Burden et al., 1983; Noakes et al., 1993; Sealock et al., 1984), and causes AChRs to co-cluster at very high density when co-expressed in heterologous cells (Froehner et al., 1990; Phillips et al., 1991).

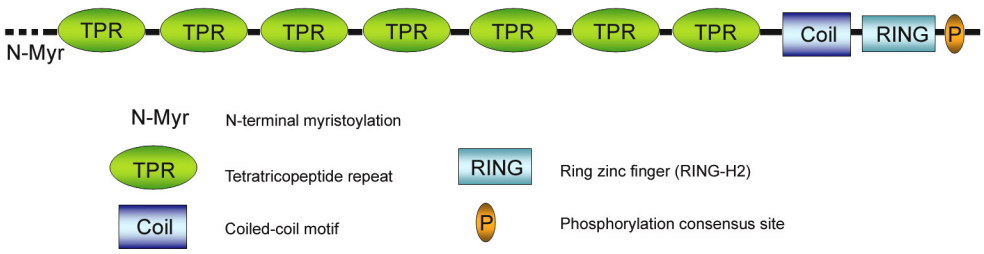


**Figure 1.6.** This overview of the structure of rapsyn shows its major domains. Rapsyn is required for agrin-induced clustering of the AChR since *rapsyn*$^{-/-}$ mice fail to cluster AChRs (adapted and modified from Willmann and Fuhrer, 2002).

Rapsyn-deficient mice fail to cluster AChRs, dystroglycan and utrophin at their mutant NMJs. Cultured *rapsyn*$^{-/-}$ myotubes are also unable to form spontaneous or agrin-induced AChR clusters (Gautam et al., 1995). This shows the central role that rapsyn plays during differentiation of the postsynaptic apparatus. Rapsyn is however placed downstream of MuSK in the agrin signalling cascade, since MuSK is still

located synaptically, and is tyrosine phosphorylated by agrin in *rapsyn*$^{-/-}$ mice (Apel et al., 1997). Presence of rapsyn is nevertheless necessary for MuSK-dependent AChR phosphorylation. This places MuSK as a principal component of a primary synaptic scaffold. This scaffold would then recruit pre-assembled AChRs via rapsyn. MuSK may be clustered in response to agrin. The extracellular domain of MuSK, apart from transducing the agrin signal, is also important in forming the primary scaffold, and forming MuSK-rapsyn links via RATL (Apel et al., 1997; Gillespie et al., 1996).

## *1.3.5 Early stages of synaptogenesis: AChR clustering*

### 1.3.5.1 Actin cytoskeleton reorganisation: regulatory pathways downstream of MuSK

Growing evidence has highlighted the participation of the actin cytoskeleton during the process of AChR clustering following agrin-induced MuSK signalling. The actin polymerisation inhibitor latrunculin A blocks agrin-induced AChR clustering, proving the importance of agrin-induced polymerisation of globular (G-) actin into filamentous (F-) actin during AChR clustering (Dai et al., 2000). Cortactin, an actin regulatory protein, is also translocated to the postsynaptic sites upon agrin stimulation (Dai et al., 2000). Two ubiquitous monomeric small GTPases of the Rho GTPase family, Rac and Cdc42, have been implicated during agrin-induced actin organisation, since they were found to be activated by agrin, and dominant interfering mutants of either of them blocked agrin-induced clustering of AChRs (Weston et al., 2000). Rac activation by agrin is also a prerequisite for Rho GTPase activation, which leads to the formation of larger macroclusters of AChRs. Rac and Rho are thus playing distinct but complementary roles in the mechanism of agrin-induced AChR clustering (Weston et al., 2003). Finn et al. (2003) have been able to show a role for members of the Abelson tyrosine kinase family (Abl) in agrin-induced AChR clustering *in vitro*. Abl kinases amplify initial agrin signalling via MuSK, and clustering of AChRs, by tyrosine phosphorylating MuSK and the AChR β subunit (see **Chapter 2**), and are thought to stabilise clusters by induction of synaptic actin network (Finn et al., 2003;

Mittaud et al., 2004). Abl kinases, and their role in postsynaptic development, are discussed in more detail below (section 1.5).

A yeast-two-hybrid screen with MuSK's cytoplasmic domain as a bait, allowed the identification of Dishevelled 1 (Dvl) (Luo et al., 2002). Dvl is a scaffolding protein discovered in *Drosophila* for its role in the development of coherent arrays of polarised cells via its activation in the Wnt/Frizzled canonical signalling pathway. Dvl possesses three conserved domains, DIX (Dishevelled-Axin), PDZ, and DEP (Dishevelled-Egl-10-Pleckstrin), and interacted with MuSK juxtamembrane and kinase motifs via its DEP domain in a phosphorylation-independent manner (Luo et al., 2002). Disrupting the MuSK-Dvl interaction inhibited agrin- and neurone-induced AChR clustering, and dominant-negative Dvl expression in postsynaptic muscle cells reduced the amplitude of spontaneous synaptic currents at the NMJ. In addition, Dvl was shown to interact with downstream serine/threonine p21-activated kinase (PAK1). Agrin activated PAK in a Dvl-dependent manner, and inhibiting PAK1 activity reduced the level of AChR clustering (Luo et al., 2002).

The Mei lab recently identified another MuSK interacting partner using double hybrid experiments. The α subunit of geranylgeranyltransferase I (GGT) was found to interact with MuSK's kinase domain (Luo et al., 2003b). Agrin induced rapid increased GGT tyrosine phosphorylation and activity of α(G/F) subunit. Inhibiting activity or expression of GGT prevented agrin-induced AChR clustering, and reduced neuromuscular synapse development in spinal neurone-muscle cocultures. *In vivo*, mice expressing an α(G/F) mutant had NMJ defects including wider endplate bands and smaller AChR pretzels. GGT catalyses the process of prenylation, which confers membrane targeting to proteins. This could be important for key components involved in the actin reorganisation following agrin stimulation such as Rac (Strochlic et al., 2005). Thereby GGT is necessary for agrin-induced AChR clustering, and formation and/or maintenance of the NMJ (Luo et al., 2003b).

The most recent player which could also play a role in the reorganisation of actin during AChR clustering is the tumour suppressor APC (Adenomatous Polyposis Coli). It is an actin-binding protein localised in the cytoplasm, nucleus and adhesive junctions. APC interacts with AChR β subunit, colocalises with AChR clusters at the

NMJ, and its functionality is required for agrin-induced AChR clustering in cultured myotubes (Wang et al., 2003). It could therefore help in the localisation of AChRs to the actin cytoskeleton (Strochlic et al., 2005).

### *1.3.6 Later stages of synaptogenesis: stabilisation of AChR clusters and maturation of the postsynaptic density*

I have so far described and outlined the steps and key players involved in the clustering of AChRs induced by agrin-MuSK signalling. However synaptogenesis proceeds beyond late embryonal stages of agrin-induced AChR cluster induction, since during the first postnatal weeks, AChR clusters are stabilised, and synaptic maturation takes place. This involves *synapse elimination*, in which multiply-innervated muscle fibres lose all but one nerve contact. This process involves activity-dependent mechanisms (Lichtman and Colman, 2000). The formation of postjunctional folds also takes place around this time, causing and allowing for concentration of certain protein complexes at the crests or in the troughs of the postsynaptic membrane, with the characteristic pretzel-shape of AChRs clustered at the crests of postjunctional folds (Marques et al., 2000).

The AChR is thought to be anchored to the actin cytoskeleton in a rapsyn-dependent process (Mohamed and Swope, 1999), with the modulatory proteins mentioned above playing important regulatory roles downstream of MuSK (Borges and Ferns, 2001). This anchoring is further brought about by members of the Src-family kinases (SFKs), since Src and Fyn activity maintains the phosphorylation the AChR β subunit required for the link of AChRs to the cytoskeleton (Sadasivam et al., 2005). Disruption of Src activity *in vivo* leads to instability and disruption of the postsynaptic density, and the absence of Src and Fyn in knockout myotubes leads to an instability in the link between AChR-rapsyn and members of the dystrophin/utrophin glycoprotein complex (D/UGC) (Sadasivam et al., 2005).

### 1.3.6.1 The D/UGC complex

The D/UGC complex links the extracellular matrix (ECM) to the muscle cytoskeleton, and mutations in members of this complex lead to muscle wasting muscular dystrophies (Campbell, 1995). There is diverse synaptic and extrasynaptic distribution of different members of the D/UGC complex (see Figure 1.3). Utrophin, β2-syntrophin and α-dystrobrevin-1 localise only at the NMJ, whilst α-syntrophin, α-dystrobrevin-2, α- and β-dystroglycan and dystrophin are found both at NMJs and extrasynaptic areas (Willmann and Fuhrer, 2002). Utrophin and dystrophin are homologous proteins, and the largest of the D/UGC complex. They are dispensable for NMJ formation, but both play a role in maintaining muscle integrity. Mice lacking utrophin only show slightly reduced AChR densities and postjunctional folds (Deconinck et al., 1997; Grady et al., 1997a). *Mdx* mice lacking functional dystrophin also have reduced postjunctional folds, indicative of muscle fibre necrosis and cycles of regeneration rather than absence of functional dystrophin (Lyons and Slater, 1991; Torres and Duchen, 1987). Both mutant mice are alive and healthy, and have functional NMJs. Doubly-deficient mice of utrophin and dystrophin however develop a severe muscular dystrophy, but NMJs still appear very normal in young animals, demonstrating that the D/UGC complex is not essential for the initial clustering of AChRs and formation of the NMJ, but vital for the maturation of the postsynaptic density, especially the postjunctional folds (Willmann and Fuhrer, 2002).

Another D/UGC complex protein, dystroglycan (DG), demonstrates early lethality in knockout mice. DG normally associates directly with rapsyn, and is colocalised with AChRs. Therefore, the lab of Carbonetto generated chimaeric mice only lacking DG in selected striated muscle fibres (Cote et al., 1999). These mice developed severe muscular dystrophy within a few months of birth, and both *in vivo* and *in vitro* DG-deficient muscle fibres showed diffuse and unstable AChR clusters (following withdrawal of agrin from culture medium) (Jacobson et al., 2001), indicative of a role for DG in the condensation and stabilisation of AChR microclusters. In addition, laminin, perlecan and acetylcholinesterase (AChE) are greatly reduced at NMJs of DG-deficient muscle (Tremblay and Carbonetto, 2006). Similarly to DG, α-dystrobrevin is also required for stabilisation of AChR clusters, as seen in

dystrobrevin mutant mice, and in myotubes from these mice whereby removal of agrin from cultures leads to rapid dispersal of agrin-induced AChR clusters (Grady et al., 1999). Therefore DG is required for D/UGC complex integrity, and recruits and anchors the complex to the muscle membrane, while dystrobrevin is needed for maintenance of clusters of AChRs and for correct postsynaptic folding and aggregation of AChRs to crests of postjunctional folds.

Tyrosine kinases also play very important roles during both agrin-induced formation of AChR clusters, as well as during stabilisation of these clusters at the NMJ. They are discussed in more detail below (see section 1.5 below). Interestingly, isoforms of dystrobrevin critical in stabilising the NMJ show tyrosine phosphorylation sites which are also of importance (Grady et al., 2003). These observations represent a link between tyrosine kinases and the D/UGC in stabilising the postsynapse of the NMJ.

## 1.4 Muscle pre-patterning: new vs. old paradigms

Recent data have emerged in the past few years challenging previously published models for how the postsynaptic apparatus assembles. Over two decades ago Harris showed that motor nerve ablation did not prevent the formation of a postsynaptic-like structure in the muscle (Harris, 1981). This observation was long underestimated but was confirmed in recent studies, in which the induction of AChR clusters *in vivo* or the positioning of aneural clusters in the central region of the muscle does not require agrin or the nerve (Lin et al., 2001; Yang et al., 2001). Indeed, it raises the possibility that the nerve terminal is actually led to grow to sites on the muscle where AChRs are pre-clustered into *hot spots*. These are then taken over, and stabilised by the arrival of the neurone (Flanagan-Steet et al., 2005). This phenomenon is termed *pre-patterning*. Postsynaptically, both MuSK and rapsyn are required for pre-patterning to occur. However neurally-released agrin is not necessarily required, since postsynaptic sites are still temporarily present in *agrin*$^{-/-}$ embryos (from E14-E18), dispersing after E18, and since subsynaptic nuclei become transcriptionally specialised even in aneural muscles (Lin et al., 2001; Yang et al., 2001).

A new model therefore is emerging on the assembly of the postsynaptic apparatus. In this model, further summarised in a very recent review by Kummer et al. (2006), pre-formed specialisations in the postsynaptic muscle membrane can participate in normal synaptogenesis, but their maintenance requires innervation. Neurotransmission, induced by the release of ACh from the presynaptic neurone, acts to restrict muscle-independent differentiation, dispersing pre-patterned postsynaptic sites (Misgeld et al., 2005). The primary role of nerve-released agrin is to counteract the local dispersal effect of synaptic activity at nerve-muscle contacts. Therefore agrin would act as a stabilisation or "anti-declustering" factor at the synapse, rather than solely as a clustering inducer (Kummer et al., 2006).

## 1.5 Roles of tyrosine kinases and phosphatases at the neuromuscular junction

During early and later stages of NMJ development, tyrosine phosphorylation plays critical roles in controlling the activation of tyrosine kinases, leading to the phosphorylation of target proteins such as the AChR, and in doing so propagating signals driving events such as clustering of AChRs. Protein tyrosine phosphatases (PTPs) are therefore essential in regulating the activity and signalling of protein tyrosine kinases (PTKs), and in controlling end levels of substrate phosphorylation, through dephosphorylation of their tyrosine residues. Tyrosine phosphorylation of some of the main players in NMJ development, such as MuSK and the AChR, has been shown to be required for proper formation of synaptic structures. Inhibition of kinase activity by means of inhibitor treatments prevents phosphorylation of the AChR β subunit, and hence agrin-induced clustering of AChRs (Ferns et al., 1996; Wallace, 1994). PTK inhibition also has effects on spontaneous AChR clusters, which are dispersed upon treatment with herbimycin or staurosporine (Ferns et al., 1996). MuSK and the AChR form an association in mouse myotubes. Agrin induces an increase in this association, as well as the tyrosine phosphorylation of both. MuSK is however not responsible for phosphorylating the AChR β subunit (Fuhrer et al., 1997). This implied that some other non-receptor tyrosine kinases must be involved in

propagating agrin-MuSK signalling to induce phosphorylation and clustering of the AChR. The main non-receptor PTKs identified so far having roles during NMJ development belong to the Abelson family of tyrosine kinases (Abl kinases) (Finn et al., 2003) and the SFKs (Fuhrer and Hall, 1996; Fuhrer et al., 1997; Mohamed and Swope, 1999). The role of PTPs at the NMJ has also been addressed, and growing evidence reveals that they play important roles in controlling phosphorylation of the AChR, its link to the cytoskeleton, agrin-induced clustering, and later stabilisation of AChR clusters (Dai and Peng, 1998; Madhavan et al., 2005; Meier et al., 1995; Wallace, 1995).

## *1.5.1 Abl kinases*

Abl1 and Abl2 (previously Abl and Arg) are the two members of the Abelson family of non-receptor PTKs (Abl kinases). The Abl kinases are characterised by a Src homology 3 (SH3) domain, Src homology 2 (SH2) domain, a tyrosine kinase domain, and unique C-terminal binding motifs for SH3 domains, DNA, filamentous F-actin, and monomeric G-actin (Figure 1.7) (Van Etten, 1999; Wang, 1993). Abl1 and Abl2 are highly conserved, localise to the plasma membrane, cytoskeleton and cytosol, they bind F-actin, and show F-actin bundling activity. Previous studies have established roles for the Abl kinases in regulating cell proliferation and survival, cytoskeletal reorganisation, cell migration, and the response to oxidative stress and DNA damage (Pendergast, 2002).

### 1.5.1.1 Abl kinases in postsynaptic development

Finn et al. (2003) have recently shown that Abl kinases localise to the postsynaptic membrane of the developing NMJ. Agrin stimulates an association between MuSK and Abl kinases, and MuSK and Abl exhibit reciprocal tyrosine phosphorylation. Inhibition of Abl kinase activity by use of the inhibitor STI571, or through the expression of a dominant-negative form of Abl1, disturbs agrin-induced clustering of AChRs. The Abl and MuSK association somewhat parallels the association between

Abl1 and EphB2 or TrkA in the CNS (Yu et al., 2001). The mode of interaction between MuSK and Abl has however not yet been established. If the interaction is direct, this could occur via the SH2 domain of the Abl kinase binding to a phosphorylated tyrosine residue on the activation loop or the juxtamembrane region of MuSK. This interaction would disinhibit the Abl kinase, subsequent phosphorylations further potentiating its catalytic activity. Alternative binding between the Abl kinase and MuSK could occur via an as yet unidentified adapter molecule (Burden et al., 2003).

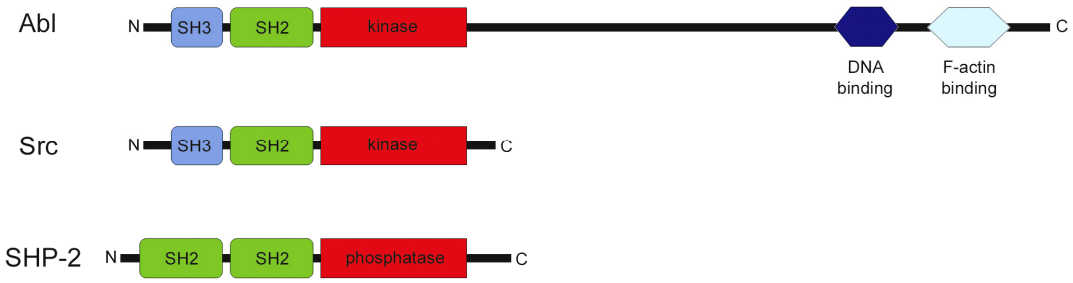


**Figure 1.7.** A schematic representation of the structures of the tyrosine kinases Abl and Src, and the tyrosine phosphatase SHP-2. Abbreviations: SH3, Src homology 3 domain; SH2, Src homology 2 domain (adapted and modified from Hubbard and Till, 2000).

The functions of the Abl kinases during the development of the NMJ could be diverse. They firstly could provide a tyrosine kinase activity downstream of MuSK, required for spatiotemporally regulating the tyrosine phosphorylation of MuSK and the AChR (see **Chapter 2**; Mittaud et al., 2004), and agrin-induced clustering of AChRs. The association between an Abl kinase and MuSK could increase the formation of a large signalling complex, and also enhance MuSK phosphorylation, allowing it to activate further downstream targets. Activation of Abl kinase by MuSK could lead to phosphorylation of known synaptic components and new effectors of cytoskeletal regulation. Finally, the Abl kinase recruitment by MuSK could concentrate the intrinsic actin bundling activity of Abl kinases at sites of postsynaptic assembly (Finn et al., 2003). The importance of actin re-organisation induced by agrin during the formation of the NMJ has already been shown, and is discussed above (Dai et al., 2000). The involvement of the Rho GTPases Rac, Rho and Cdc42 in regulating actin

organisation and in agrin-induced clustering of AChRs in myotubes has been shown by Weston and colleagues (2003; 2000). PAK1 is an effector of Rac and Cdc42 in actin reorganisation, and is linked to MuSK via Dishevelled 1 (Dvl) upon agrin induction (Luo et al., 2002). Abl kinase could therefore link MuSK stimulation with Rac/Cdc42/PAK1 in regulating the actin cytoskeleton in AChR clustering.

### *1.5.2 Src-family kinases*

Src-family kinases (SFKs) comprise a family of related non-receptor PTKs containing an N-terminal SH3 domain (which binds polyproline type-II (PPII) helical structures), an SH2 domain (which binds phosphotyrosine (pY) sequences), and a C-terminal kinase domain (Figure 1.7) (Chong et al., 2005). SFKs were initially thought to be involved in regulation of cell proliferation and differentiation because Src, the prototype member of the family, was first identified as a proto-oncogene (Stehelin et al., 1976). They were however found to be ubiquitously expressed, and have multiple cellular functions such as cytoskeletal alterations, differentiation, survival, adhesion, and migration (Thomas and Brugge, 1997).

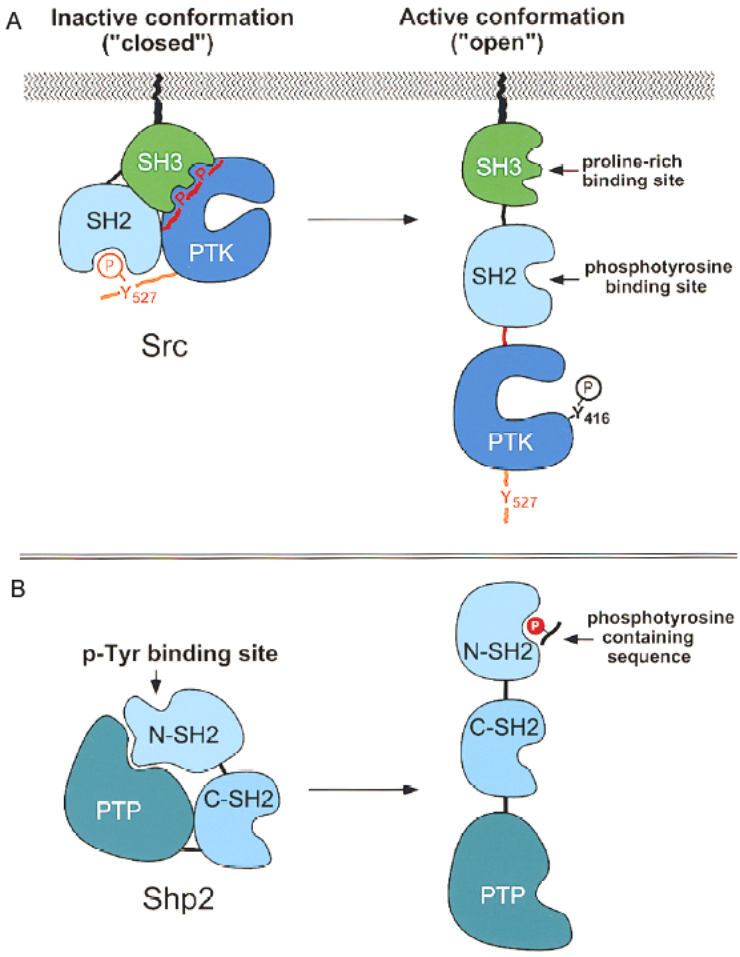


**Figure 1.8.** Regulation of the enzymatic activity of Src and SHP-2. Autoinhibition occurs via an intramolecular mechanism. In Src, this regulation occurs via SH3- and SH2-mediated autoinhibition. In SHP-2 the SH2-mediated inhibition controls its phosphatase activity (adapted from Weiss and Schlessinger, 1998).

### 1.5.2.1 Regulation of Src activity

The activity of Src is regulated via an intramolecular negative regulatory mechanism (Figure 1.8A). Src possesses a tyrosine at its C-terminus which provides a negative regulatory phosphorylation site (Thomas and Brugge, 1997). The crystal structure of Src has revealed that its kinase domain is maintained in an inactive state by two different intramolecular interactions with its SH3 and SH2 domains (Xu et al., 1997). The SH3 domain binds to a short proline type-II helix in a region connecting the SH2 domain to the PTK domain (SH2-kinase linker), and the SH2 domain binds to the C-terminal $pY_{527}$. Release of Src autoinhibition and stimulation of Src tyrosine kinase activity may be brought about in one of three ways. A first possibility is the binding of

target proteins containing proline-rich sequences to the SH3 domain. Alternatively binding of phosphotyrosine-containing motifs to the SH2 domain also releases Src from its *closed* inactive conformation. A third way is via the dephosphorylation of $pY_{527}$ at the C-terminus of Src. The resulting *open* conformation of Src renders it ready for phosphorylation of $Y_{416}$ on its activation loop (A loop) via intermolecular interaction with another Src molecule (Weiss and Schlessinger, 1998).

### 1.5.2.2 SFKs in postsynaptic development

SFK members Fyn and Fyk were found to be major PTKs in the electric organ of *Torpedo californica* (Swope and Huganir, 1993), and to associate with AChRs via their SH2 domains (Swope and Huganir, 1994). In C2C12 (C2) myotubes, Src and Fyn are associated with AChRs (Fuhrer and Hall, 1996), Src can phosphorylate MuSK and the AChR β subunit upon agrin stimulation (Mohamed et al., 2001), and AChR-bound SFKs are activated and tyrosine phosphorylated in agrin treated C2 myotubes (Mittaud et al., 2001). Staurosporine abolishes this activation, as does the absence of rapsyn in *rapsyn*$^{-/-}$ myotubes (Mittaud et al., 2001). MuSK also forms a complex with Src and Fyn in C2 myotubes, and the SFKs in turn phosphorylate MuSK early on but not later following agrin stimulation (see Chapter 2; Mohamed et al., 2001). *In vivo* analysis of the importance of Src and Fyn for postsynaptic development showed that in *src*$^{-/-}$;*fyn*$^{-/-}$ mice, synaptic development occurs normally (innervation, gene expression, and clustering of postsynaptic proteins) until birth, after which the animals die (Smith et al., 2001). Clustering of AChRs and phosphorylation of the AChR β subunit in agrin-bathed myotubes derived from these *src*$^{-/-}$;*fyn*$^{-/-}$ mice is also unaffected by the absence of these SFKs. There is however an upregulation of a third member of the family, Yes, which could to some extent compensate for the absence of Src and Fyn (Smith et al., 2001). Importantly, the agrin-induced AChR clusters of myotubes deficient in Src and Fyn are far less stable than wild-type myotubes following the withdrawal of agrin from the cultures (Smith et al., 2001). Furthermore, very recent *in vivo* evidence for the role of SFKs came when Sadasivam and colleagues showed that SFKs stabilise the NMJ via protein interactions, phosphorylation, and cytoskeletal linkage of AChRs (Sadasivam et al., 2005).

Therefore SFKs clearly play an important role at the NMJ. They are important in the temporal control of agrin-induced MuSK and AChR β subunit phosphorylation, and in establishing an AChR stabilisation programme that acts much later after agrin induction (see **Chapter 2**; Mittaud et al., 2004). Finally, Src and Fyn are dispensable for AChR clustering, but are clearly required to stabilise agrin-induced AChR clusters (Sadasivam et al., 2005), showing that the mechanism for formation versus stabilisation of these clusters are different (Willmann and Fuhrer, 2002).

### *1.5.3 Protein tyrosine phosphatases*

Protein tyrosine phosphatases (PTPs) are enzymes that dephosphorylate tyrosine residues on proteins which have initially been phosphorylated by PTKs. PTPs comprise a large superfamily of proteins that can be further classified into *classical* PTPs and *dual-specificity* PTPs (DSPs). The classical PTPs contain one or more phosphatase domains consisting of a conserved stretch of ~240 residues containing the unique signature motif (I/V)HCxxAGxxR(S/T)G. The structure of the active site of the classical PTP, particularly the active site cleft depth, renders it specific to phosphotyrosine residues (over phosphoserine or phosphothreonine). Classical PTPs can be subdivided into transmembrane, receptor-like (RPTPs), and non-transmembrane (non-TM) PTPs (Neel and Tonks, 1997; Paul and Lombroso, 2003; Tonks and Neel, 2001).

PTPs exert both positive and negative effects on signalling pathways (Van Vactor et al., 1998), and several human diseases have been identified to be caused by mutations in a PTP (van Huijsduijnen et al., 2002). PTPs play important roles in attenuating signals generated by PTKs. In fact, the inhibition of PTPs using the potent and specific inhibitor sodium pervanadate leads to an enhancement in the PTK-mediated tyrosine phosphorylation (Gordon, 1991; Posner et al., 1994). In the presence of pervanadate leading to the inhibition of PTP activity, the resulting basal PTK activity is often sufficient to induce cellular responses, which would otherwise be absent under normal PTP activity. These responses can either be positive or negative,

therefore leading to PTPs having both positive as well as negative regulatory roles in PTK signalling pathways (Hunter, 1995).

### 1.5.3.1 Regulation of PTP activity

A general mechanism for regulation of phosphatase activity in non-receptor PTPs is via blockade of their catalytic site. As with the regulation of SFKs, an intramolecular blockade may be the principal mode of regulation. The Src homology 2 domain-containing phosphatase 2 (SHP-2) contains two N-terminal SH2 domains, and a phosphatase domain at its C-terminal end (Figure 1.7). The crystal structure of SHP-2 reveals how the N-terminal SH2 domain interacts with the catalytic cleft of the PTP domain via an intramolecular interaction, thereby autoinhibiting SHP-2 (Hof et al., 1998). A loop of the SH2 domain occupies the catalytic site, preventing binding of phosphotyrosine-containing motifs to the PTP domain, and holding SHP-2 in an inactive *closed* conformation (Figure 1.8B). The SH2 domain phosphotyrosine-binding site, not involved in the interaction with the catalytic core, is free for interaction with appropriate phosphotyrosine-containing substrates. The binding of a phosphopeptide to this site causes a change in the conformation of SHP-2 by destabilising the interaction of the N-SH2 with the catalytic cleft, liberating it from its *closed* state to an *open* state, and releasing it from autoinhibition. Therefore, upon recruitment of SHP-2 by its substrate, at the right cellular location, and at the right time, the phosphatase activity of SHP-2 is available for action (Denu and Dixon, 1998; Tonks and Neel, 2001; Weiss and Schlessinger, 1998).

### 1.5.3.2 PTPs in postsynaptic development

The role played by PTPs during postsynaptic development has only recently been addressed. The main strategies used in addressing the role of PTPs have been pharmacological approaches, used in combination with biochemical and molecular techniques. The potent PTP inhibitor sodium pervanadate, and other PTP inhibitors, combined with *in vitro* and cell culture-based techniques, have given insight into how PTPs are actively controlling processes such as phosphorylation of MuSK and of the

AChR, and during agrin- and growth factor-independent and agrin- and growth factor-induced aggregation of AChRs on the muscle membrane.

Work in Bruce Wallace's lab has shown that the treatment of myotubes with pervanadate has several consequences. Firstly, the phosphotyrosine content of postsynaptic proteins and of AChRs is increased, and agrin-induced clustering of AChRs is prevented by pervanadate treatment (Meier et al., 1995; Wallace, 1995). Secondly, phosphatase inhibitor treatment slows down the extractability of AChRs (as does agrin), and decreases the lateral mobility of diffusely distributed spontaneous AChRs along the muscle membrane surface. Thirdly, agrin increases the rate of change of phosphorylation of AChR β subunit induced by pervanadate, and also increases the rate of extractability of AChRs. These findings show that tyrosine phosphorylation regulates formation and stability of AChR clusters by strengthening the link of AChRs to the (actin) cytoskeleton, and that agrin activates PTK activity, which drives a local signal for clustering of AChRs (Meier et al., 1995; Wallace, 1995). This agrin-induced PTK activity drives tyrosine phosphorylation events leading to clustering of AChRs, and stabilising clustered receptors to the cytoskeleton (Madhavan and Peng, 2005). The fact that mobility of AChRs is reduced throughout myotubes as a result of increased phosphorylation following pervanadate treatment shows that active PTKs and PTPs are continuously involved in controlling the phosphorylation status of AChRs (Meier et al., 1995). If PTPs are blocked and AChRs become hyperphosphorylated, the receptors are locally linked to the cytoskeleton and cannot be clustered by agrin (Wallace, 1995).

H.B. Peng's lab has also addressed the question on the role of PTPs in postsynaptic development. They have shown that PTPs mediate dispersal of AChR clusters. Agrin-stimulated MuSK activates PTKs which promote AChR clustering, while concomitantly activating downstream PTPs which dephosphorylate and dissolve AChRs from pre-existing clusters (or *hot spots*), allowing them to redistribute. Madhavan et al. (2005) have also shown that low level inhibition of PTPs by means of pervanadate does not immobilise AChRs completely, and causes auto-activation of MuSK combined with an increase in spontaneous AChR clustering. Moreover, agrin-induced clusters appear larger. In nerve-muscle co-cultures, or using growth factor-coated beads, the induced AChR clusters have less discrete boundaries upon PTP

inhibition, shedding light on the importance of PTP activity locally, at the site of nerve- or growth factor-induced MuSK signalling (Dai and Peng, 1998; Madhavan and Peng, 2005). Peng therefore proposes a scheme, based on the Alan Turing model, whereby short-range activation signals and long-range inhibition signals combine to generate a spatiotemporally regulated system. Therefore he proposes that agrin activates MuSK, leading to high PTK activity locally at the site of stimulation, driving a positive feedback loop stimulating MuSK. The balance between PTK and PTP activity therefore lies in favour of the former, keeping MuSK phosphorylated and active. MuSK activation would also lead to PTP activation which would control the level of clustering of AChRs further downstream of the site of stimulation, keeping it low, and dispersing aneural AChR clusters (Dai and Peng, 1998; Madhavan and Peng, 2003; Madhavan and Peng, 2005; Madhavan et al., 2005).

#### 1.5.3.3 SHP-2

Insight into the identities of the PTKs and PTPs stimulated by agrin and MuSK has been given in several studies. Abl kinases and SFKs are now known to be phosphorylating both MuSK and the AChR β subunit upon agrin stimulation, as discussed above (see Chapter 2; Finn et al., 2003; Mittaud et al., 2004; Mohamed et al., 2001; Swope and Huganir, 1993). As for the identity of the PTP, a phosphatase was purified from *Torpedo* electric organ and found to associate with and to dephosphorylate the AChR (Mei and Huganir, 1991). Mei and colleagues, using a PCR strategy, identified SHP-2. SHP-2 occurs abundantly in muscle and colocalises with the AChR on the postsynaptic density of the NMJ *in vivo* (Mei and Si, 1995; Tanowitz et al., 1999). Very recently, SHP-2 was depleted in C2 myotubes using siRNA (small-interfering RNA). This led to an enhancement of spontaneous and agrin-induced AChR clustering (Madhavan et al., 2005). This places SHP-2 as a very good candidate as a PTP controlling PTK activity during events leading to the development of the postsynaptic density of the NMJ.

SHP-2 is a cytoplasmic PTP that may be activated through association of its N-SH2 domain to phosphotyrosine residues on proteins such as membrane receptors (Figure 1.8B). This allows for strict spatiotemporal regulation of its phosphatase activities,

and ensures that SHP-2 is active at the right place at the right time. SHP-2 could thus be recruited to the postsynaptic density by a RTK such as MuSK upon agrin-induced MuSK phosphorylation, or by another downstream PTK such as an Abl kinase or a SFK. Recruitment would hence allow its activation and participation in balancing the kinase activity present, and establish the right level of phosphorylation required for stable signalling. I have addressed the function of SHP-2 in C2 myotubes in culture, in the clustering of AChRs into hot spots, during agrin-induced clustering, and in the stability of agrin-induced AChR clusters following agrin withdrawal (see **Chapter 3**).

## 1.6 Aims of this thesis

In this thesis I have sought to delve deeper into the signalling mechanisms important during the formation and stabilisation of AChR clusters at the neuromuscular synapse. The receptor tyrosine kinase MuSK plays a central role during these signalling pathways, and relays messages driven by neurally-released agrin to the muscle, instructing and driving the clustering of AChRs. Subsequent phases rely on the stabilisation of these clusters by securing a link between the AChRs and the actin cytoskeleton. Apart from MuSK, other tyrosine kinases are known to be activated by agrin, and required for propagating the agrin signal by activating MuSK and the AChR, and controlling the intensity and development of the agrin signal. Tyrosine phosphatases are important in regulating phosphorylation events and therefore, in conjunction with PTKs, govern the outcome of signalling pathways. I therefore studied the role played by non-receptor PTKs and PTPs in the agrin signalling pathway leading to AChR clustering, and in the stabilisation of these clusters in the absence of agrin.

In the introductory chapter of this dissertation, **Chapter 1**, I have given an overview of the signalling mechanisms involved during the formation of the neuromuscular synapse, as well as molecular processes responsible for stabilisation and maturation of the synapse. I have also given insight into the most recent hypotheses on the mechanisms of NMJ formation, highlighting what is known to date about the roles played by PTKs and PTPs at the synapse. In **Chapter 2**, taken from an article

published in Molecular and Cellular Biology (Peggy Mittaud, Alain A. Camilleri, Raffaella Willmann, Susanne Erb-Vögtli, Steven J. Burden, and Christian Fuhrer, 2004), I show how SFKs and Abl kinases control temporal aspects of agrin signalling. I also show that agrin rapidly induces a signalling cascade through activation of a kinase, which acts over a long period of time autonomously of agrin to cluster AChRs by maintaining activation of MuSK. The role played by tyrosine phosphatases in the clustering of AChRs has not been thoroughly investigated in the literature. I therefore sought, in **Chapter 3**, to establish the importance of a balance between kinase and phosphatase activity during the signalling leading to spontaneous clustering and the agrin-induced clustering of AChRs, as well as in the stability of these agrin-induced clusters after removal of agrin. I also shed new light onto the identity of a phosphatase, SHP-2, which plays important roles during all stages of agrin signalling and that is required for stability of agrin-induced AChR clusters. The data from this chapter is ready to be submitted for publication (Alain A. Camilleri, Matthias Gesemann and Christian Fuhrer). Finally, in my concluding chapter, **Chapter 4**, I discuss the implications of my findings regarding the activities of PTKs and PTPs in signalling pathways leading to AChR clustering, and in stabilising these clusters thereafter, and the requirement of a fine balance of their activities to achieve a stable synapse. I draw parallels to related mechanisms at other synapses, including those in the CNS. I also briefly outline human diseases affecting the NMJ, in particular focusing on autoimmune diseases such as myasthaenia gravis (MG).

# Chapter 2

## *A single pulse of agrin triggers a pathway that acts to cluster acetylcholine receptors*

This chapter has been adapted from an article originally published in **Molecular and Cellular Biology** (Peggy Mittaud*, Alain A. Camilleri*, Raffaella Willmann, Susanne Erb-Vögtli, Steven J. Burden, and Christian Fuhrer, Mol. Cell. Biol. Vol. 18, pp. 7841-7854, 2004).

* These authors contributed equally.

My contributions to the paper were many of the biochemical experiments, for which I developed concepts, performed the experimental procedures, and wrote the corresponding sections. Examples are the different temporal actions of SFKs versus Abl kinases or the determination of the amounts of MuSK protein in cultured myotubes.

## 2.1 Abstract

Agrin triggers signalling mechanisms of high temporal and spatial specificity to achieve phosphorylation, clustering and stabilisation of postsynaptic acetylcholine receptors (AChRs). Agrin transiently activates the kinase MuSK; MuSK activation has largely vanished when AChR clusters appear. Thus a tyrosine kinase cascade acts downstream from MuSK, as illustrated by the agrin-evoked long-lasting activation of Src-family kinases (SFKs) and their requirement for AChR cluster stabilisation. We have investigated this cascade and report, first, that pharmacological inhibition of SFKs reduces early but not later agrin-induced phosphorylation of MuSK and AChRs, while inhibition of Abl kinases reduces late phosphorylation. Interestingly, SFK inhibition applied selectively during agrin-induced AChR cluster formation caused rapid cluster dispersal later, upon agrin withdrawal. Second, a single 5-min agrin pulse, followed by extensive washing, triggered long-lasting MuSK and AChR phosphorylation and efficient AChR clustering. Following the pulse, MuSK phosphorylation increased and, beyond a certain level, caused maximal clustering. These data reveal novel temporal aspects of tyrosine kinase action in agrin signalling. First, during AChR cluster formation, SFKs initiate early phosphorylation and an AChR stabilisation program that acts much later. Second, a kinase mechanism rapidly activated by agrin acts thereafter autonomously in agrin's absence, to further increase MuSK phosphorylation and cluster AChRs.

## 2.2 Introduction

Neuromuscular synapses are cellular contacts of remarkable specialisation. The presynaptic terminal is specialised to release neurotransmitter on demand, and the postsynaptic membrane, by accumulating a high density of clustered acetylcholine receptors (AChRs) and associated proteins, is specialised to generate an endplate potential of sufficient magnitude to reliably initiate an action potential in the muscle. To assemble and maintain these structures, it is essential that pre- and postsynaptic cells exchange signals to coordinate their differentiation in time and space.

One such signalling exchange is initiated by agrin, a nerve-derived signal that is concentrated in the synaptic basal lamina (McMahan, 1990). Agrin rapidly stimulates MuSK, a receptor tyrosine kinase of skeletal muscle, and agrin/MuSK signalling is essential for the formation of neuromuscular synapses (Gautam et al., 1996; Glass et al., 1996). Little is known about the mechanisms by which agrin activates MuSK and how MuSK activation leads to pre- and postsynaptic differentiation. Phosphorylation of tyrosine residues in the MuSK activation loop and the juxtamembrane region, however, is essential for agrin-induced clustering of AChRs (Herbst and Burden, 2000; Zhou et al., 1999). Interestingly, agrin-induced activation of MuSK is transient and has largely vanished by the time AChR clusters appear (Fuhrer et al., 1997). This suggests that a downstream pathway is activated and raises the issue of whether such a pathway operates autonomously in the absence of continuous agrin stimulation.

Pharmacological studies indicate that MuSK stimulation indeed activates a downstream tyrosine kinase cascade important in clustering of AChRs (Fuhrer et al., 1997). Within this cascade Src-family kinases (SFKs), which are associated with AChRs (Fuhrer and Hall, 1996), become phosphorylated and activated rapidly by agrin treatment, and their activation lasts much longer than the activation of MuSK (Mittaud et al., 2001). A crucial player in the cascade are Abl kinases, as they are required for AChR clustering, associate with MuSK and phosphorylate MuSK (Finn et al., 2003). The downstream cascade also leads to tyrosine phosphorylation of the AChR β and δ subunits (Mittaud et al., 2001; Wallace et al., 1991), and

β phosphorylation is required for efficient clustering and cytoskeletal interaction of the AChR (Borges and Ferns, 2001). It remains unclear which kinase phosphorylates the AChR in response to agrin, although SFKs have been implicated (Mohamed et al., 2001; Smith et al., 2001), and it is unknown if the downstream kinase cascade requires continuous agrin stimulation to remain active and lead to AChR clustering.

Following their formation in embryogenesis, neuromuscular synapses become structurally and functionally mature during early postnatal life. Although agrin/MuSK signalling is likely to have a role in synaptic maturation, additional signalling mechanisms may regulate synaptic maturation and maintenance without having an essential role in synapse formation (reviewed by Huh and Fuhrer, 2002; Sanes and Lichtman, 2001; Willmann and Fuhrer, 2002). Separate pathways for synapse formation and synapse maintenance/maturation are illustrated in mice lacking utrophin and dystrophin or in mice lacking α-dystrobrevin, a component of the dystrophin/utrophin-glycoprotein complex; in these mice, neuromuscular synapses form but fail to mature properly (Grady et al., 1997b; Grady et al., 2000). Notably, in the absence of α-dystrobrevin, synaptic AChR clusters are normal at birth but increasingly fragment postnatally, indicating a defect in the mechanisms that stabilize the postsynaptic membrane (Grady et al., 2000). Similarly, in cultured myotubes lacking α-dystrobrevin, AChR clusters form normally in response to agrin stimulation, but these clusters are unstable and disperse rapidly when agrin is withdrawn from the myotubes (Grady et al., 2000). α-Dystrobrevin acts at least in part via tyrosine phosphorylation of the α-dystrobrevin-1 isoform, suggesting the involvement of a tyrosine kinase in postsynaptic stabilisation (Grady et al., 2003).

Good candidates for such a kinase are SFKs. Previously, we analysed mice that were mutant for Src and Fyn, Src and Yes or Fyn and Yes and found that neuromuscular synapses appear normal in mice lacking these pairs of SFKs (Smith et al., 2001). SFKs, however, are important for stabilizing AChR clusters, as AChR clusters, which form normally in cultured myotubes lacking both Src and Fyn, are unstable following the withdrawal of agrin and rapidly fragment into microclusters in these mutant cells (Smith et al., 2001). Similarly, Src and Fyn stabilize AChR clusters that were induced by laminin, indicating their importance in more than one clustering pathway (Marangi et al., 2002). Together, these observations raise the question of whether the activation

of SFKs upon agrin stimulation is linked with the role of SFKs in AChR cluster stabilisation.

To elucidate the temporal action of tyrosine kinases in agrin signalling, we have investigated how the downstream kinase cascade acts in clustering and stabilisation of AChRs. We find that upon agrin stimulation, SFKs phosphorylate the AChR and MuSK early but not later, and that SFKs recruit a pathway for stabilisation of AChR clusters already during initiation of cluster formation. Furthermore, we find that a single 5 min agrin pulse, followed by efficient washing, is sufficient to trigger maximal AChR clustering, first detectable several hours following the pulse, indicating that agrin rapidly activates an autonomous pathway that subsequently runs in the absence of continual agrin stimulation.

## 2.3 Materials and Methods

### *2.3.1 Expression of agrin and cell culture*

Soluble neurally-derived agrin (C-$Ag_{12,4,8}$) was produced in COS cells as previously described (Fuhrer et al., 1997). Cell culture reagents were purchased from Life Technologies (Basel, Switzerland). C2C12 (C2), *src*$^{-/-}$*;fyn*$^{-/-}$ myoblasts (clones DM11 and DM15) and the corresponding wild-type myoblasts (clones SW5 and SW10) were grown and fused to form myotubes as previously described (Fuhrer et al., 1997; Smith et al., 2001). Most of the experiments were done with clones SW5 and DM11 but the other clones gave identical results.

### *2.3.2 Antibodies and inhibitors*

Antibodies against phosphotyrosine (PY20, 4G10), the AChR γ and δ subunits (mAb88), the conserved C-terminus of Src, Fyn and Yes (src-CT), against MuSK, against the AChR β subunit (mAb124) or the AChR α subunit (mAb35), were all used as previously described (Marangi et al., 2001; Mittaud et al., 2001). Sequence-specific phosphorylation of SFKs (at $Y_{215}$, $Y_{418}$, or $Y_{529}$) was detected by rabbit polyclonal phosphopeptide-specific antibodies purified by sequential epitope-specific chromatography as performed by the supplier (BioSource Europe, S.A., Belgium). These antibodies were generated against the phosphorylation sites and flanking peptides of Src but are expected to crossreact with Fyn and Yes based on the high sequence conservation. Rabbit polyclonal antibodies raised against the C-terminus of agrin were used in Western blots are detailed previously (Sugiyama et al., 1994). To analyze the effect of anti-agrin antibody mAb33 on clustering, agrin was pre-incubated for 1 h at room temperature with the antibody (0.025 - 0.1 mg/ml) and then applied to myotubes for 6 h. In experiments with brief agrin pulses, agrin was added for 5 min. Agrin was withdrawn, cells were washed with medium, and incubated with media containing mAb33. mAb33 was generated by Dr. Werner Hoch (University of

Houston) (Hoch et al., 1994) and commercially available through Stressgen Biotechnologies (Victoria, Canada).

To examine the role of SFKs in agrin-induced AChR phosphorylation and clustering, a novel, potent and SFK-selective tyrosine kinase inhibitor, CGP77675 (kindly provided by Dr. M. Susa, Novartis, Switzerland) was used (Missbach et al., 1999). Myotubes were pre-incubated for 90 min in medium containing 10 µM CGP77675, stimulated with agrin in the presence of inhibitor and subjected to precipitation assays or immunocytochemical stainings as described below. For AChR clustering assays, we occasionally re-added inhibitor (10 µM) several times during agrin incubation to ensure its full activity. We obtained the same results whether or not we re-added CGP77675. CGP77675 had no significant effect on spontaneous AChR clustering nor on cell morphology. In controls, CGP77675 was omitted and the carrier (DMSO) was used alone. 10 µM of CGP77675 is an established concentration for use in cell cultures (Missbach et al., 1999; Recchia et al., 2003) and 250-fold greater than the $IC_{50}$ for Src autophosphorylation in vitro and 500-2000-fold greater than the $IC_{50}$ for phosphorylation of peptide substrates in vitro (Missbach et al., 1999). We also used a range of concentrations of inhibitor, from 3 µM to 30 µM, for AChR clustering and phosphorylation and obtained similar results as for 10 µM, although effects were a bit smaller at 3 µM.

To block Abl kinases, C2 myotubes were treated with 10 µM STI 571 (Finn et al., 2003) analogous to CGP77675. We also used 20 µM STI 571 and obtained the same results.

### *2.3.3 Precipitation assays and immunoblot analysis*

To examine the effects of CGP77675 on agrin-induced phosphorylations, myotubes were pre-treated for 90 min with the inhibitor, followed by 0.5 nM agrin for 5 min or 40 min in the presence of CGP77675. Cell lysates were split into two parts and analysed either by MuSK or AChR precipitation followed by phosphotyrosine immunoblotting as previously described (Mittaud et al., 2001). To precipitate AChRs, biotin-coupled α-BTX was used, followed by streptavidin-coupled agarose beads

(Mittaud et al., 2001). Phosphotyrosine bands were identified based on their molecular weight and by reprobing immunoblots with antibodies reactive with the AChR subunits or with SFKs (src-CT). MuSK was precipitated using polyclonal anti-MuSK antibodies and protein A-sepharose (Fuhrer et al., 1997). Quantitation was performed by densitometric scanning as described previously (Mittaud et al., 2001). Mostly signals from cells not treated with agrin were used as 100% control. For some Figures, signals of cells treated with agrin for 5 min were used as 100% controls, because in untreated cells signals were sometimes too small or even undetectable to serve as a reference. In all cases tyrosine phosphorylation of AChRs, AChR-associated SFKs and of MuSK were clearly increased by 5 min agrin treatment in comparison to untreated cultures. In all quantitations of tyrosine phosphorylation of AChRs or AChR-associated proteins (using α-BTX-precipitation), signals were normalized for the amount of precipitated AChRs, as revealed by reprobing blots for the AChR α or γ and δ subunits. Effects of STI 571 on phosphorylation were studied in an analogous way.

### *2.3.4 Single brief agrin pulse*

Myotubes were stimulated with 0.1 or 0.5 nM agrin for 5 or 40 min, washed twice with fusion medium containing no agrin and incubated with medium lacking agrin. After the appropriate times, 8 h for clustering, AChR phosphorylation and clustering were analysed. To analyze the role of SFKs, myotubes were pre-treated with CGP77675 and stimulated with agrin followed by agrin withdrawal, all in the presence of CGP77675.

### *2.3.5 Quantitation of agrin and MuSK*

To quantify cell-bound agrin per mg of cellular protein, lysates made from agrin-treated myotubes were analysed by agrin-immunoblotting. Using an agrin standard, we calculated cell-bound agrin (fmole) per mg of cellular myotube protein in the lysates loaded onto the SDS-gel. The smallest detectable signal (2.4 fmole/mg), which

represent the detection limit of this procedure, indicates the maximal amount of agrin that may remain cell-bound after a 5 min pulse and withdrawal (Figure 2.8A). Quantitation of agrin was performed three times, with similar results.

To estimate the amount of MuSK per mg of cellular protein, MuSK-antibodies known to work properly in precipitation assays (Fuhrer et al., 1997) were covalently coupled to protein A-Sepharose beads using dimethyl pimelimidate (Harlow and Lane, 1999). As control, pre-immune serum was coupled. Myotube lysates, typically containing 12 mg cellular protein, were incubated with MuSK antibodies and beads pelleted. Pellets, containing precipitated MuSK, and supernatants, containing soluble MuSK, were boiled in SDS-PAGE-buffer. Two steps were performed to estimate MuSK. First, 90% of pellets were subjected to SDS-PAGE and SYPRO Ruby staining, a highly sensitive luminescent protein stain of superior linearity ideal for quantitation of a wide range of proteins (Molecular Probes, Eugene, OR) (Berggren et al., 2000). Precipitated MuSK, identified based on its molecular weight and absence in the pre-immune control precipitation, was quantified by blue-light illumination and densitometric scanning using a parallel bovine serum albumin (BSA) standard. Typically, 100 ng of MuSK was found in these precipitates. Second, 10% of pellets and 2% of supernatants were analysed by MuSK-immunoblotting and densitometric scanning, revealing that typically 60% of total MuSK in the original myotube lysate was precipitated, while 40% remained in the supernatant. This estimation of MuSK was performed three times and yielded an average amount of 179.1 ± 74.6 (mean ± SEM) fmole MuSK / mg cellular protein. Although based on an indirect method of MuSK quantitation, this estimation implies that the amount of agrin remaining myotube-associated after withdrawal is only ca. 1% of the level of MuSK.

### *2.3.6 Immunocytochemical staining, fluorescence microscopy and quantitation of clusters*

AChRs were visualised by incubating myotubes with 100 nM tetramethylrhodamine-conjugated α-BTX (Molecular Probes) in fusion medium for 1 h at 37 °C followed by fixation (Marangi et al., 2001). Cells were mounted in glycerol containing

p-phenylenediamine (Sigma) to reduce fluorescence fading and were examined with a fluorescence microscope (Axioskop 2, Carl Zeiss, Germany). To quantitate clusters, 10-20 random fields of myotubes taken at 400x magnification were chosen by phase contrast, and signals were counted as clusters as described previously (Marangi et al., 2001) i.e., if signal intensity was clearly distinguishable from diffuse staining and strongly above background levels, and if signals had an elongated shape. Within each experiment, the number of AChR clusters per myotube was averaged. Data from at least three such experiments were used to calculate mean ± SEM.

## 2.4 Results

### *2.4.1 Agrin increases phosphorylation of the main autophosphorylation site in AChR-associated SFKs*

In our initial experiments, we sought to determine how activation of Src and Fyn, bound to the AChR, is regulated. A hallmark of SFK activation is phosphorylation of a tyrosine in the activation loop, $Y_{418}$, which represents the main autophosphorylation site (Figure 2.1A) (Thomas and Brugge, 1997). In addition, dephosphorylation of a C-terminal phosphotyrosine, $Y_{529}$, leads to maximal and sustained activity. Dephosphorylation of $Y_{529}$ displaces the intramolecular $pY_{529}$ – SH2-domain-interaction, enhancing kinase reconfiguration and activity (Thomas and Brugge, 1997). Thus, by monitoring $Y_{418}$ phosphorylation, we can assess SFK activation, while the status of $Y_{529}$ is an indication of the strength and duration of activation.

We monitored phosphorylation of AChR-associated SFKs by treating C2 myotubes with agrin and precipitating AChRs (Tox-P) using α-bungarotoxin (α-BTX) as reported previously (Mittaud et al., 2001). We stimulated myotubes with 0.5 nM agrin for 40 min, because phosphorylation of MuSK, SFKs and AChRs are maximal at this time (Fuhrer et al., 1997; Mittaud et al., 2001). As shown by immunoblotting, overall tyrosine phosphorylation of AChR-bound SFKs was indeed increased by agrin (Figure 2.1B). Probing Western blots with phosphopeptide-specific antibodies to either $pY_{418}$, $pY_{215}$ or $pY_{529}$ in SFKs, we found that $Y_{418}$ phosphorylation increases 2.8-fold following agrin stimulation (Figure 2.1C). In contrast, phosphorylation of $Y_{215}$, residing within the SH2-domain, decreased 1.7-fold, while agrin did not diminish phosphorylation of $Y_{529}$ (Figure 2.1C). These changes in phosphorylation at individual tyrosine residues correspond well with the overall 2.2-fold increase in tyrosine phosphorylation of SFKs induced by agrin (Mittaud et al., 2001) (see also Figure 2.1B) and suggest that this overall increase largely reflects the increased phosphorylation of $Y_{418}$. Taken together, agrin stimulates phosphorylation at the activation loop tyrosine $Y_{418}$ in AChR-associated SFKs, indicating their activation.

Since $pY_{529}$ is not dephosphorylated, this activation appears submaximal and sustained by other mechanisms.

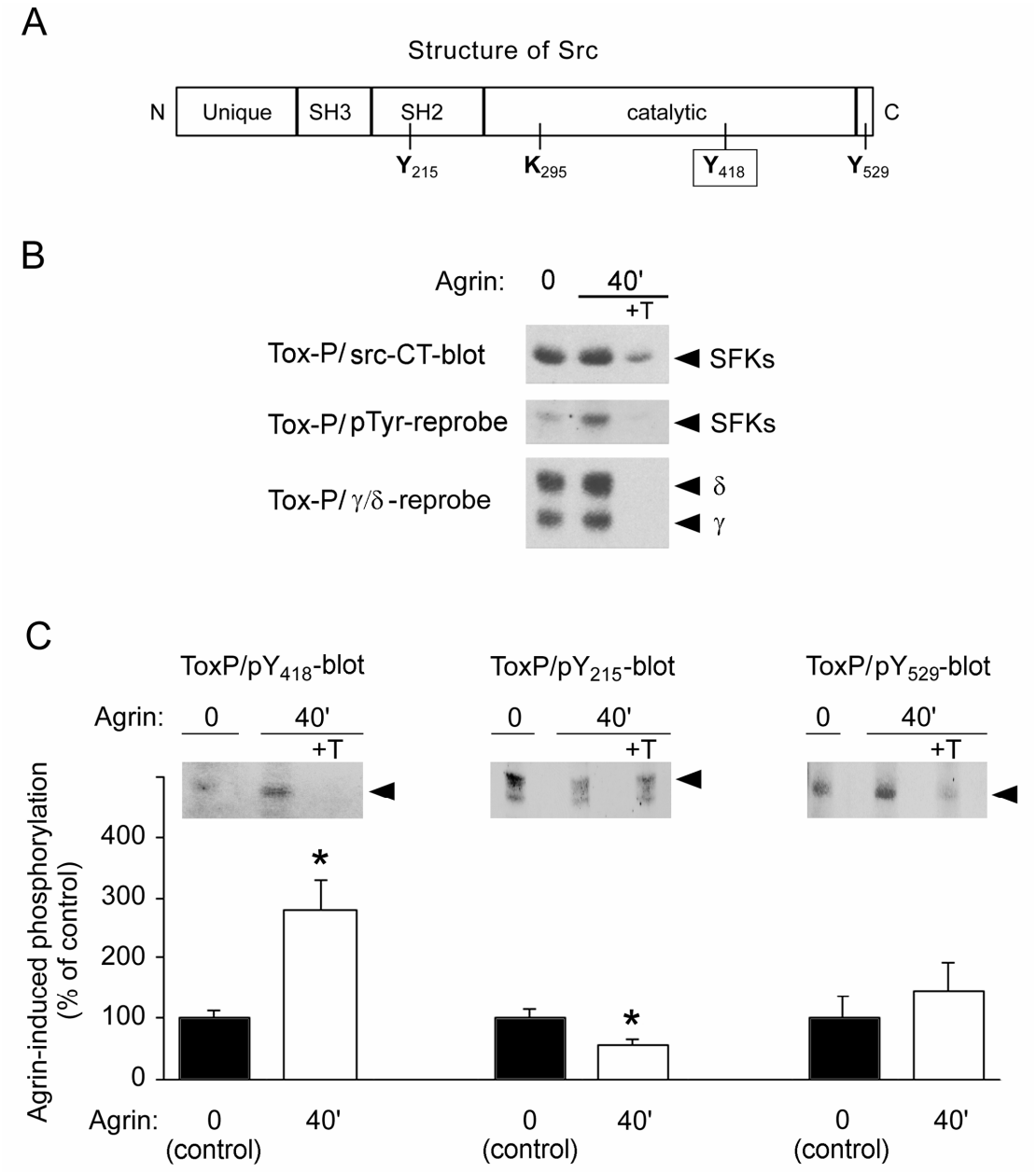


**Figure 2.1. Agrin increases phosphorylation of $Y_{418}$ in AChR-associated SFKs.** (A) A cartoon showing the position of tyrosine residues ($Y_{215}$, $Y_{418}$, $Y_{529}$) important in regulation of SFKs. (B) From C2 myotubes treated with 0.5 nM agrin, AChRs were precipitated using biotin-α-BTX (Tox-P), followed by immunoblotting. Controls included addition of 10 μM free α-BTX (+T). The blot was probed with pan-Src antibodies (src-CT), followed by anti-phosphotyrosine and AChR (γ- and δ-subunit) reprobing. (C) Blots were probed with antibodies against $Y_{418}$, $Y_{215}$ and $Y_{529}$ phosphopeptides, and bands originating from SFKs (arrows; identified by src-CT reprobing) were quantitated. Background signals from control precipitations (+T) were subtracted from AChR precipitations to yield quantitations (mean ± SEM of at least 4 experiments). * Differs significantly from non-agrin-treated control ($p<0.05$, by two-tailed paired t-test).

### *2.4.2 Early but not later agrin-induced phosphorylation is inhibited by CGP77675, a SFK-selective tyrosine kinase inhibitor*

In addition to phosphorylation of AChR-associated SFKs, agrin induces tyrosine phosphorylation of AChR β and δ subunits (Mittaud et al., 2001), raising the possibility that the AChR may be a direct substrate for SFKs. To test this idea, we blocked SFK activity by treating myotubes with 10 μM CGP77675, a potent and SFK-specific inhibitor. We stimulated myotubes, pre-treated with inhibitor, with agrin for 5 min, because phosphorylation is first detectable at this time (Fuhrer et al., 1997), and for 40 min. CGP77675 was continuously present during the agrin incubation, and we analysed α-BTX-AChR-precipitates by phosphotyrosine immunoblotting (Figure 2.2). We observed that AChR β subunit-phosphorylation, induced by a 5 min agrin treatment, is inhibited by CGP77675 whereas β subunit phosphorylation, induced by a 40 min treatment, is not affected (Figure 2.2A). Although these experiments indicated that SFKs are ultimately dispensable for stimulating AChR β subunit phosphorylation, we were concerned that the failure of CGP77675 to inhibit this phosphorylation at 40 min may be due to a loss of CGP77675 activity at this time. We therefore determined whether the inhibitor remained active during the agrin incubation by measuring the level of total cellular tyrosine phosphorylation in myotube lysates. Figure 2.2B shows that phosphorylation was equally reduced in CGP77675-treated myotubes incubated with agrin for 5 or 40 min. Thus, CGP77675 is equally efficient at reducing total cellular tyrosine phosphorylation at 5 and 40 min but only effective in inhibiting AChR β subunit phosphorylation at 5 min following agrin stimulation. Furthermore, we used higher CGP77675 concentrations, up to 30 μM, in these assays and obtained the same results (P. Mittaud and C. Fuhrer, unpublished observations).

Because AChR β phosphorylation was greater after 40 min of agrin treatment than after 5 min (Figure 2.2A), we were concerned that our assay might not detect an inhibitory effect of CGP77675 at 40 min. We therefore stimulated myotubes with 20 nM rather than 1 nM agrin, which induced strong AChR β phosphorylation at 5 min, similar to the β phosphorylation observed at 40 min using 1 nM agrin. We found that CGP77675 nonetheless blocks this strong early, but not the late β phosphorylation

(Figure 2.2C). Thus, SFK activity is required for early but not later tyrosine phosphorylation of the AChR β subunit.

We next studied the effect of CGP77675 on agrin-induced phosphorylation of AChR δ subunits, AChR-associated SFKs and MuSK. Quantitation showed that early (5 min), but not late (40 min) phosphorylation of AChR δ, AChR-bound SFKs and MuSK are inhibited by 10 μM (Figure 2.2D, E, F) or 30 μM CGP77675 (data not shown). To exclude the possibility of non-specific inhibition of other, non-Src-family kinases by CGP77675, we used this inhibitor also at lower concentrations, down to 3 μM, in phosphorylation experiments of AChR β, MuSK and cellular proteins. We found similar effects as those shown in Figure 2.2A, B and F. Furthermore, we used another SFK inhibitor, PP2 (10 μM) (Smith et al., 2001), in such assays and again obtained comparable results (P. Mittaud and C. Fuhrer, unpublished observations).

Together, these data indicate that SFK activity is important for phosphorylation of AChR β and δ subunits, of SFKs and of MuSK within the first 5 min of agrin stimulation but not after 40 min of agrin stimulation. Thus, these proteins may be direct substrates for agrin-activated SFKs at this early step, whereas later, they are likely phosphorylated by other kinases. Interestingly, this also applies for SFKs themselves, since they are inactivated by CGP77675 at 40 min agrin treatment as judged by impaired substrate phosphorylation (Figure 2.2B), while their own overall phosphorylation is not affected (Figure 2.2E). Thus, under these particular conditions of CGP77675 treatment, SFKs, although catalytically inactive, may be phosphorylated by other kinases, and one good candidate for such a kinase is MuSK, because MuSK interacts with SFKs (Mohamed et al., 2001).

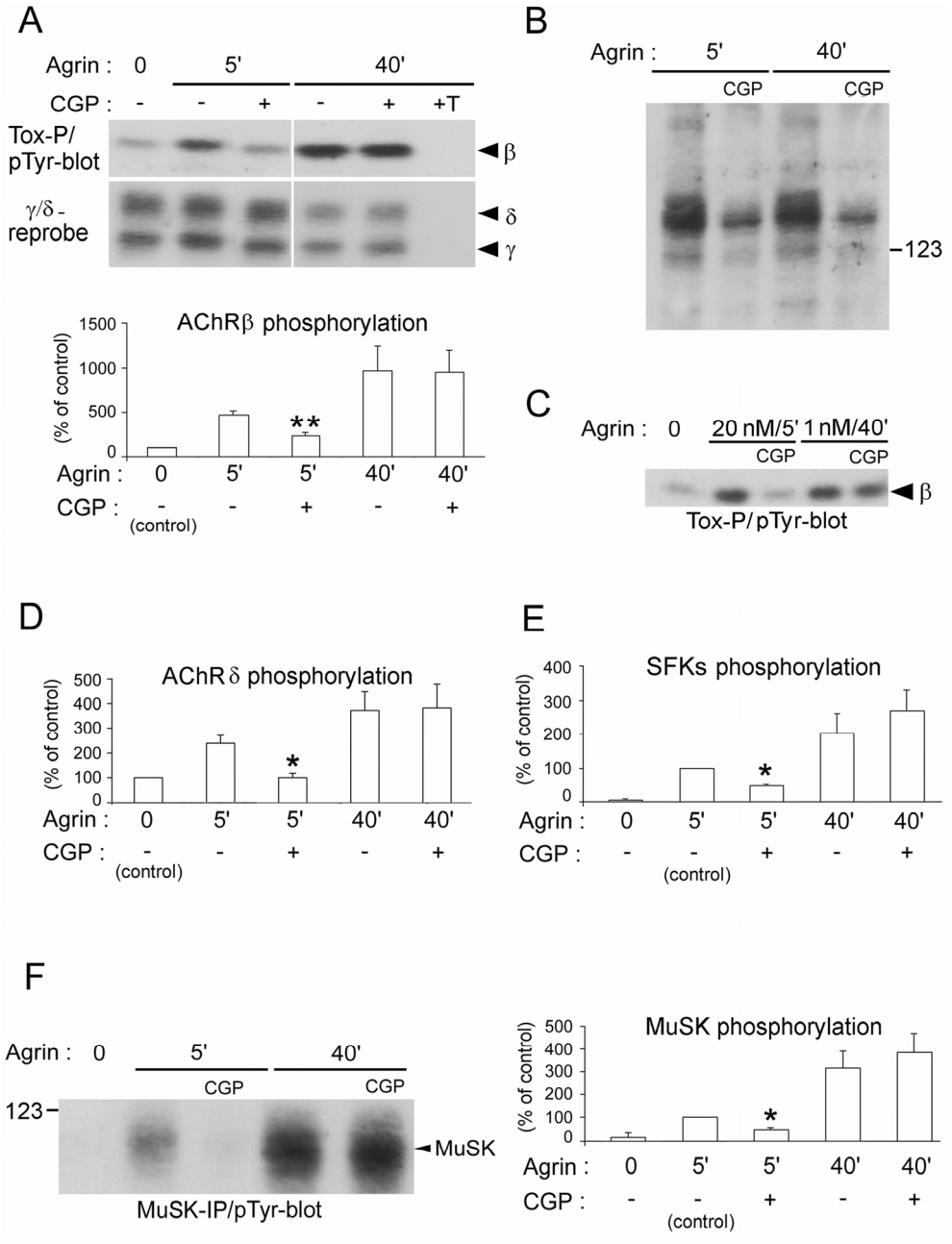


**Figure 2.2. CGP77675 inhibits early but not late phosphorylation induced by agrin.** C2 myotubes were treated with 1 nM agrin in the presence or absence of 10 μM CGP77675, and processed by α-BTX- (A, C, D, E) or MuSK- (F) precipitation and phosphotyrosine immunoblotting. Proteins of interest were identified based on their molecular weight and reprobing (not shown). (A) Quantitation of AChR β phosphorylation shows that CGP77675 inhibits early (5 min) but not late (40 min) β phosphorylation. (B) As control for CGP77675 efficiency, total lysates were analysed by phosphotyrosine immunoblotting. The reduction in phosphorylation of cellular proteins shows that CGP77675 is active and inhibits SFKs. (C) Even when using 20 nM agrin to obtain strong AChR β phosphorylation after 5 min, CGP77675 blocks this early, but not late (1 nM agrin for 40 min) phosphorylation. (D, E and F) Densitometric quantitation shows that CGP77675 inhibits early but not late phosphorylation of AChR δ subunits, AChR-associated SFKs and MuSK. All quantitations represent mean ± SEM of at least 4 experiments. * Differs significantly from 5 min agrin-treatment without inhibitor, $p<0.05$ (**$p<0.005$), by two-tailed paired t-test.

### *2.4.3 In absence of Src and Fyn, AChRs but not MuSK are phosphorylated by SFKs early in agrin signalling*

Cultured myotubes contain three major SFKs: Src, Fyn and Yes (Fuhrer and Hall, 1996). To analyze whether Src and Fyn are the critical SFKs that mediate early agrin-induced phosphorylation, we examined myotubes derived from mice lacking both Src and Fyn (*src*$^{-/-}$;*fyn*$^{-/-}$ myotubes)(Smith et al., 2001). Figure 2.3A shows that even in these cells, a 5 min agrin treatment increases phosphorylation of AChR β subunits similar to wild-type or C2 myotubes (see Figure 2.2A for comparison). As the SFK Yes is upregulated and appears to associate with AChRs in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes (Smith et al., 2001), Yes could be responsible for such phosphorylation. We therefore applied CGP77675, which efficiently inhibits Yes (Missbach et al., 1999), to these cells and found that it indeed inhibited early but not late AChR β subunit phosphorylation (Figure 2.3A). Thus, in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes, early AChR β phosphorylation requires a SFK, most likely Yes, whereas later β phosphorylation involves a non-SFK.

Interestingly, CGP77675 inhibited early MuSK phosphorylation in wild-type but not in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes (Figure 2.3B). This shows that in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes Yes cannot substitute for Src and Fyn in mediating MuSK phosphorylation and that a non-SFK is responsible for early and late MuSK phosphorylation in these mutant cells. Furthermore, overall levels of MuSK phosphorylation appear slightly higher in the mutant (Figure 2.3B). This suggests that SFKs may regulate MuSK in both positive and negative ways: if all SFKs are present (wild-type situation), their activity is required for early MuSK phosphorylation; if Src and Fyn are missing (*src*$^{-/-}$;*fyn*$^{-/-}$ situation), MuSK phosphorylation occurs more efficiently, is independent of SFK activity and may occur from MuSK itself or from Abl (Finn et al., 2003). Thus Src and Fyn, by interacting with MuSK (Mohamed et al., 2001), may normally hinder MuSK and/or Abl from phosphorylating MuSK maximally. In the absence of Src and Fyn, MuSK and/or Abl activity may become upregulated to phosphorylate MuSK to a higher degree. The complex of MuSK, Abl, Src and Fyn may allow fine-tuning of the phosphorylation status of MuSK, which is a central regulator in postsynaptic assembly.

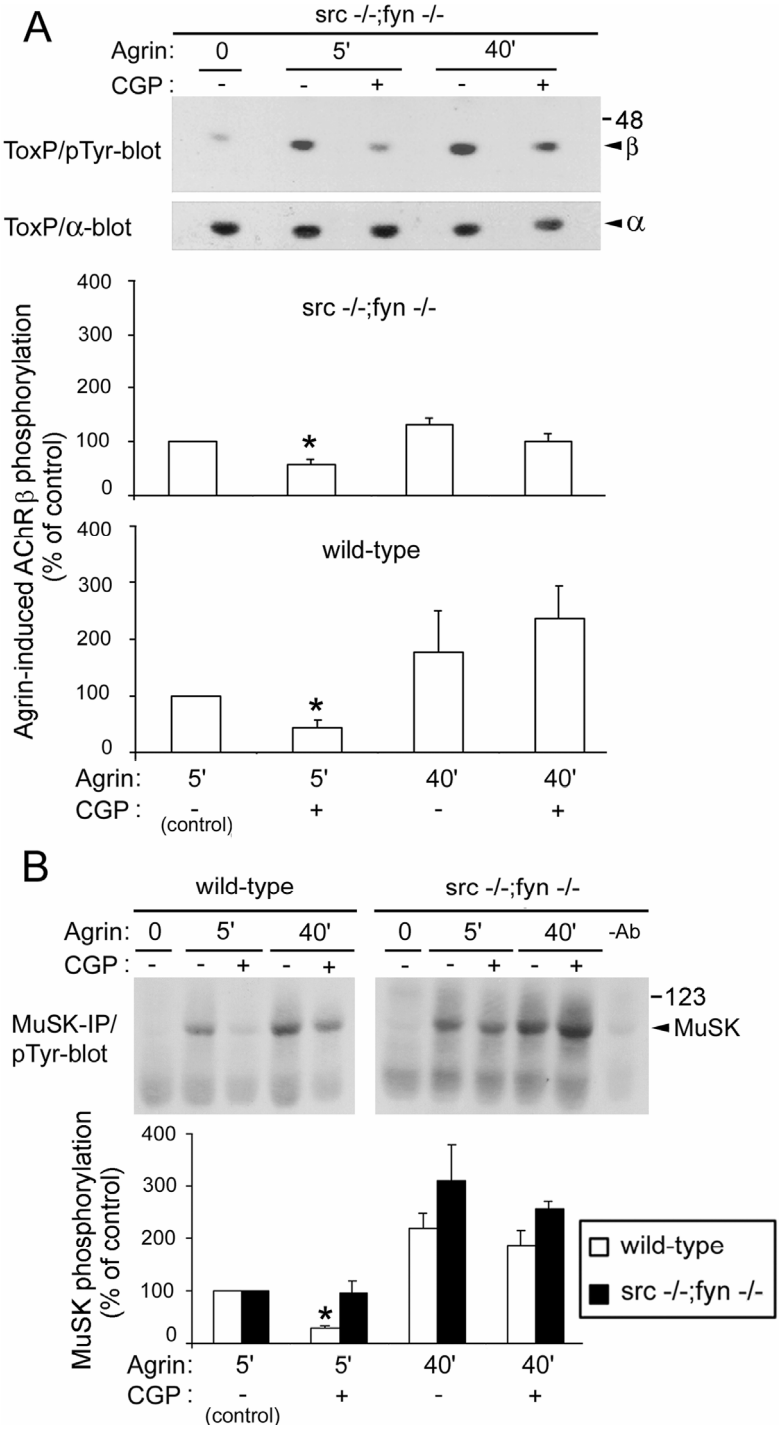


**Figure 2.3. In myotubes lacking Src and Fyn, early AChR β subunit phosphorylation induced by agrin is inhibited by CGP77675, while MuSK phosphorylation is not affected.** (A) *Src*$^{-/-}$*;fyn*$^{-/-}$ and wild-type myotubes were treated with 1 nM agrin and CGP77675. Phosphorylation of AChR β subunits was analysed, followed by reprobing for the AChR α subunit. (B) MuSK was precipitated from wild-type and *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes and analysed by phosphotyrosine immunoblotting. As control, the MuSK antibody was omitted (-Ab). For all quantitations, which reflect mean ± SEM of at least 3 experiments, signals of cells treated with agrin for 5 min without inhibitor were set to 100%. * Differs significantly from 5 min agrin-treatment without CGP77675 ($p<0.05$ in A; $p<0.006$ in B; by one-sample student's t-test).

### *2.4.4 Abl kinases mediate late phosphorylation of AChRs and MuSK*

Since Abl kinases are required for AChR clustering (Finn et al., 2003), we investigated whether they cause phosphorylation of AChRs and MuSK later in agrin signalling. As before for CGP77675, we pre-treated C2 myotubes with 10 μM STI 571, a specific Abl inhibitor (Finn et al., 2003), and added agrin for 5 or 40 min in the continuous presence of STI 571. Phosphorylation of AChR β subunits and of MuSK was substantially reduced at 40 min of agrin treatment (Figure 2.4). At 5 min, AChR β phosphorylation was slightly reduced while MuSK was not affected (Figure 2.4). These data show that Abl kinases preferably act to phosphorylate AChRs and MuSK later (40 min) in agrin signalling, in contrast to SFKs, which cause phosphorylation early (5 min) in agrin signalling.

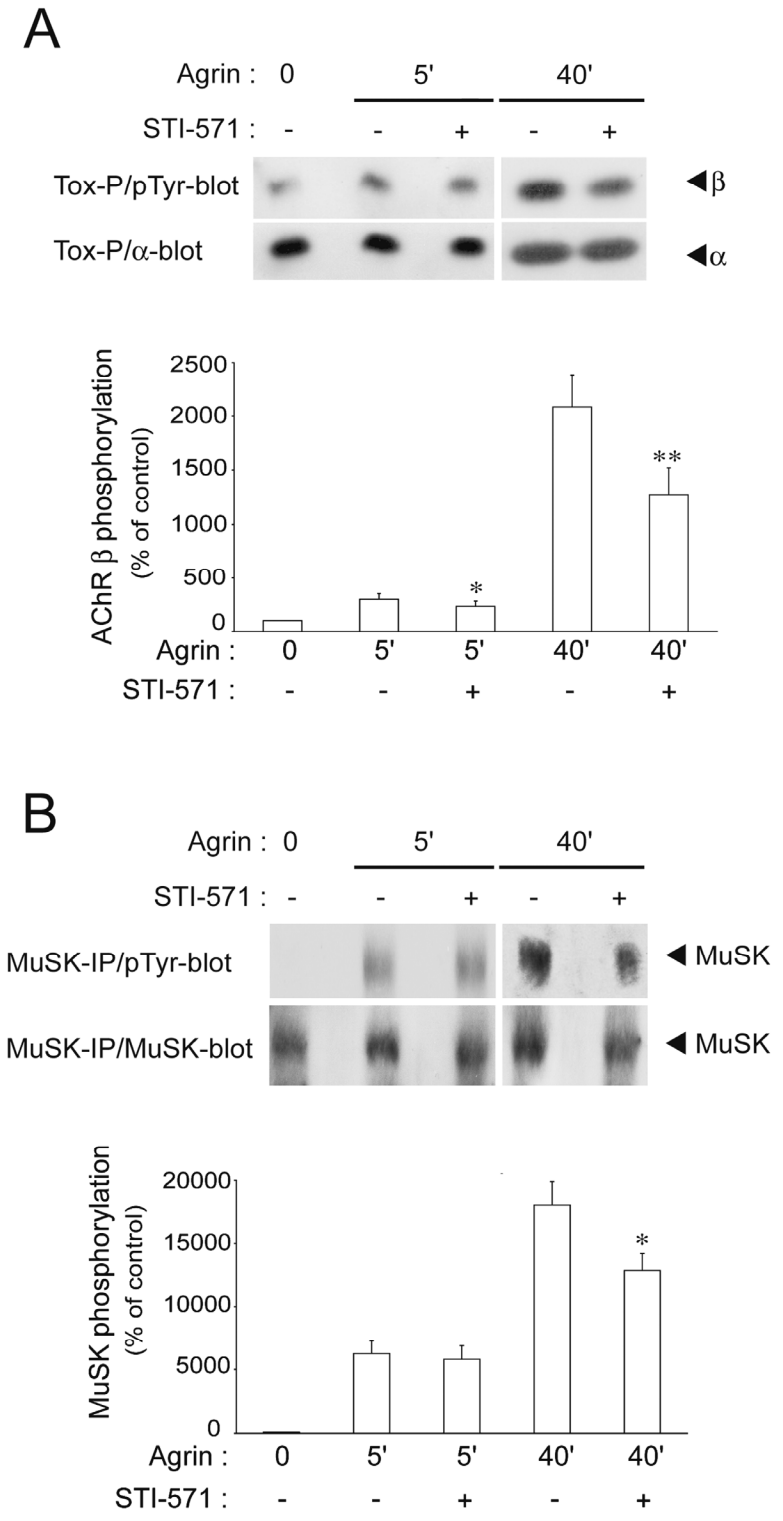


**Figure 2.4. STI 571 inhibits late AChR β and MuSK phosphorylation induced by agrin.** C2 myotubes were treated as in Figure 2.2, but STI 571 was used to inhibit Abl kinases. Phosphotyrosine blots were reprobed with antibodies against AChR α subunits (A) or MuSK (B). Quantitation (mean ± SEM from at least 8 experiments) reveals a pronounced reduction by STI 571 in phosphorylation of AChR β subunits and MuSK at 40 min of agrin treatment (** significant difference from corresponding inhibitor-free sample, $p<0.01$ (*$p<0.05$), by two-tailed paired t-test).

### *2.4.5 Inhibition of SFKs during the formation of AChR clusters leads to a defect in the stability of AChR clusters*

To investigate how SFK activation relates to AChR clustering, we first examined the effect of CGP77675 on the formation of clusters induced by agrin. As reported previously for other SFK inhibitors, PP1 and PP2 (Smith et al., 2001), 10 μM CGP77675 had no discernable effect on the overall number, length, shape or intensity of AChR clusters induced by agrin in C2, wild-type myotubes and *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes (Figure 2.5A). We observed the same results when we re-added CGP77675 several times during agrin treatment or when we used higher concentrations (30 μM; data not shown). Thus, SFK activity is not required for agrin-induced AChR cluster formation.

Once induced by agrin, AChR clusters are remarkably stable and disperse only slowly ($t_{1/2}$ = 10 - 20 h) following withdrawal of agrin (Smith et al., 2001). The stability of these AChR clusters is dependent upon SFKs: in myotubes lacking Src and Fyn, agrin-induced AChR clusters disperse rapidly ($t_{1/2}$ = 80 – 120 min) following agrin withdrawal (Smith et al., 2001). We therefore asked whether the agrin-stimulated phosphorylation of SFKs, which is maximal after 40 min (Figure 2.1) (Mittaud et al., 2001), may have a role in stabilizing AChR clusters, which form hours later.

For this purpose, we treated myotubes with CGP77675 and agrin, followed by withdrawal of both CGP77675 and agrin. AChR clusters formed normally, but then rapidly dispersed upon withdrawal, with a $t_{1/2}$ of about 3 h (Figure 2.5B). In contrast, when CGP77675 was present during the withdrawal but not the agrin induction period, the stability of AChR clusters was normal (Figure 2.5C). These findings demonstrate that SFKs act during the agrin induction period to stabilize AChR clusters hours later after agrin withdrawal (Figure 2.5B). In addition, these results indicate that the subsequent resumption of SFK activity, during the agrin withdrawal period, is insufficient to stabilize AChR clusters that formed while SFKs were inhibited. We verified this resumption by measuring total tyrosine phosphorylation of

cellular proteins by immunoblotting and found it to be normal at the end of the withdrawal period (data not shown).

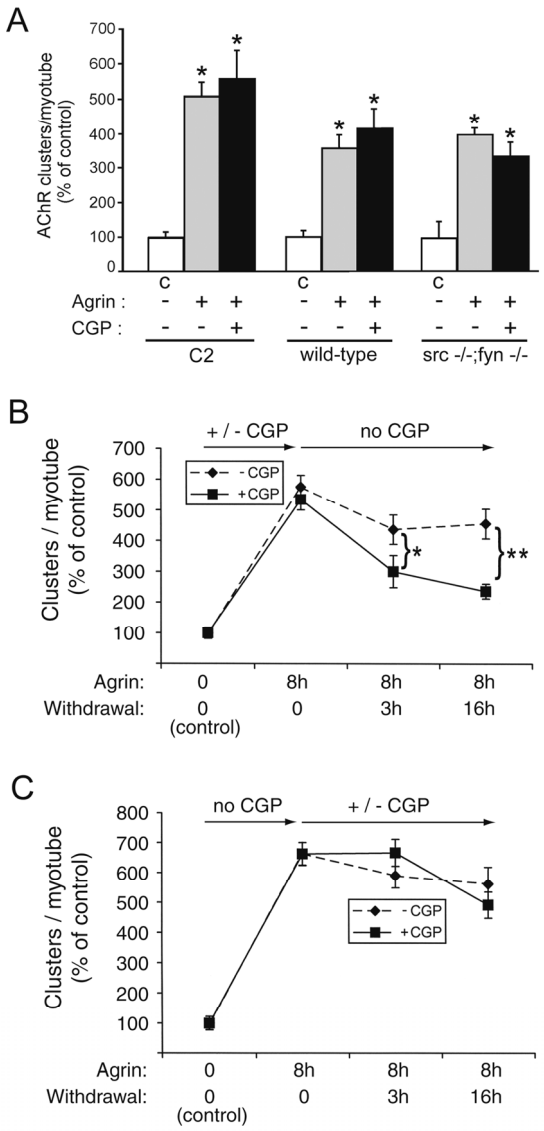


**Figure 2.5. SFK activity during cluster formation is required for cluster stabilisation later on**. (A) Myotubes were stimulated overnight with 0.5 nM agrin in the presence or absence of CGP77675, stained with rhodamine-α-BTX and analysed by fluorescence microscopy. AChR clustering was quantitated and shown as % of untreated cells (C, control; mean ± SEM of at least 3 experiments). * Differ significantly from the respective untreated cells ($p<0.03$), but not from each other ($p>0.09$, by two-tailed paired t-test), showing that CGP77675 does not inhibit agrin-induced formation of AChR clusters. (B) C2 myotubes were treated with 0.5 nM agrin for 8 h in the presence (squares) or absence (diamonds) of CGP77675, followed by withdrawal (for 3 or 16 h) of both CGP77675 and agrin, as indicated by arrows. AChR clustering was quantitated as in A, showing no difference in the number of clusters formed, but the clusters dispersed more rapidly in CGP77675-treated cells (*$p<0.03$; **$p<0.001$; by two-tailed paired t-test). (C) C2 myotubes were stimulated 8 h with agrin, followed by withdrawal of agrin and addition (squares) or no addition (diamonds) of CGP77675 into the agrin-free medium. After 3 or 16 h of withdrawal, AChR clustering was quantified as in A, revealing no differences between CGP77675-treated and untreated cells ($p>0.2$).

### *2.4.6 A single brief agrin pulse is sufficient to trigger long-lasting phosphorylation of MuSK and AChR β*

Agrin (0.5 nM) induces phosphorylation of MuSK and AChRs within 5 min (Fuhrer et al., 1997) (Figure 2.2). In the continual presence of agrin, these early phosphorylations increase substantially, ca. 4-fold, to reach a peak after 40 min (Fuhrer et al., 1997) (see also Figure 2.9). 6-8 hrs later, when AChR clusters have formed, MuSK phosphorylation has largely vanished but is still easily detectable, while AChR phosphorylation stays relatively high (Fuhrer et al., 1997). Thus, agrin triggers a long-lasting downstream tyrosine kinase activity (Mittaud et al., 2001), and ultimately leads to clustering of AChRs and many other proteins after several hours. It remains unknown whether all of these aspects are initiated within the first minutes of agrin action, whether thereafter agrin's presence is still required or whether the downstream cascade, once initiated, is self-sustaining and operates autonomously.

Therefore we studied the consequences of a brief agrin treatment. C2 myotubes received a single pulse of agrin (0.5 nM) for 5 or 40 min, followed by withdrawal of agrin, extensive washing, and incubation in agrin-free medium for 8 h (Figure 2.6A). The level of AChR β subunit phosphorylation following the brief pulse was compared to the level of phosphorylation induced by continuous agrin stimulation for 8 h. Surprisingly, β phosphorylation was the same in all of these treatments, showing that rapid agrin-induced AChR β phosphorylation is maintained long after agrin has been withdrawn (Figure 2.6A). Similar results were obtained for MuSK: a single brief agrin pulse (5 min) was sufficient to induce long-lasting MuSK phosphorylation, equivalent to that found in myotubes treated continuously with agrin for 8 h (Figure 2.6B). These results demonstrate that agrin acts during 5 min to stimulate a signalling pathway that thereafter is maintained and runs autonomously in the absence of further agrin stimulation.

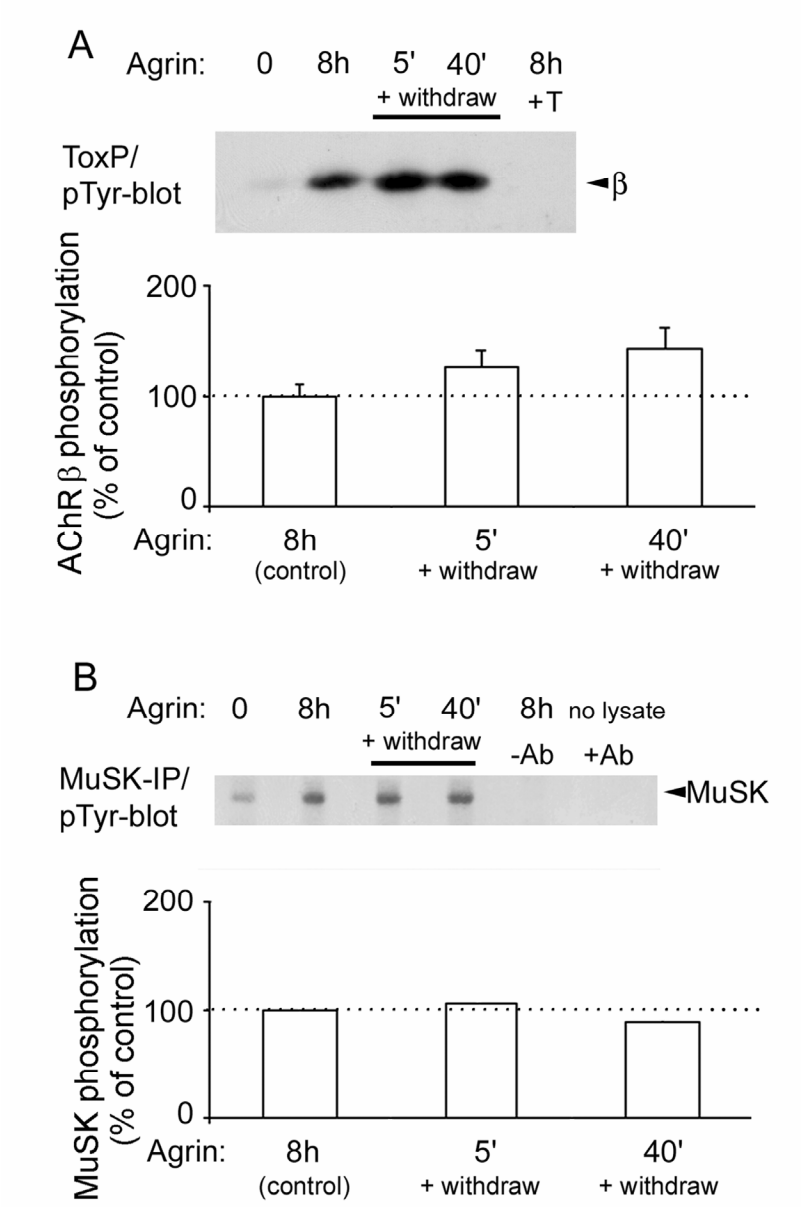


**Figure 2.6. A single 5 min agrin pulse triggers long-lasting MuSK and AChR β phosphorylation.** C2 myotubes were treated for 8 h continuously with 0.5 nM agrin. Parallel cultures were incubated for 5 min or 40 min with 0.5 nM agrin, followed by agrin withdrawal, washing and incubation in agrin-free medium for 8 h. All lysates were then split into two parts and analysed either by AChR- (A) or MuSK- (B) precipitation, followed by phosphotyrosine immunoblotting. As controls, we added a 10 µM free α-BTX (+T; A) or omitted the lysate or the MuSK-antibody (-Ab; B). (A) Quantitation represents the mean ± SEM of 3 experiments and shows no significant differences between the pulsed or continuous agrin treatments ($p>0.63$). (B) Quantitation represents the mean of two experiments that showed very small variations between each other.

### *2.4.7 A single brief agrin pulse leads to efficient AChR clustering*

We examined whether this autonomous pathway, rapidly triggered by agrin, also leads to normal AChR clustering. For this purpose we added agrin for 5 or 40 min, withdrew agrin, washed the cells extensively, incubated them in agrin-free medium, and quantitated AChR clusters 8 h later. We observed no significant differences in the number, size or density of AChR clusters between the pulsed 5 min or 40 min and the continuous 8 h treatment with agrin (Figure 2.7). We further investigated whether this AChR clustering is dependent on SFK activity by applying CGP77675. The inhibitor had no significant effect on agrin-induced AChR clustering, even when this clustering was induced by a single brief pulse of agrin, as short as 5 min (Figure 2.7B). Thus, a brief agrin treatment triggers a mechanism that runs autonomously, in the absence of further agrin stimulation, to cause long-lasting MuSK and AChR phosphorylation and efficient AChR clustering. This clustering, like continuous agrin treatment, is independent of SFK activity.

It was important to establish that our agrin withdrawal protocol removed the vast majority of cell-bound agrin and that clustering and phosphorylation were not caused by small amounts of agrin potentially remaining after withdrawal. We performed two control experiments to address whether agrin-related clustering activity binds to cells within a 5 or 40 min pulse. First, we stimulated myotubes with 0.5 nM agrin for 5 or 40 min and then transferred the agrin-containing supernatant to a separate myotube culture. Since 0.5 nM of soluble agrin is below saturation for AChR clustering and phosphorylation (Ferns et al., 1996), a decrease in the concentration of this agrin should reduce AChR clustering and phosphorylation. We found, however, that the transferred supernatant induced AChR clustering and β subunit-phosphorylation as efficiently as agrin that had not been exposed to myotubes (P. Mittaud and C. Fuhrer, unpublished observations). Thus, as no detectable agrin-activity was absorbed from agrin-containing media within 5 or 40 min, these data imply that very little agrin binds to myotubes within that time frame. Second, agrin was applied to cells for 5 or 40 min, and withdrawn for 8 h. After the 8 h, the withdrawal media was transferred to other myotubes, where it failed to induce AChR clustering or β phosphorylation (P. Mittaud and C. Fuhrer, unpublished observations). Thus, after a brief agrin pulse,

followed by withdrawal, insufficient agrin is present in the withdrawal medium to stimulate AChR clustering, implying that only low levels of agrin, if any, remain attached to myotubes after the pulse and wash.

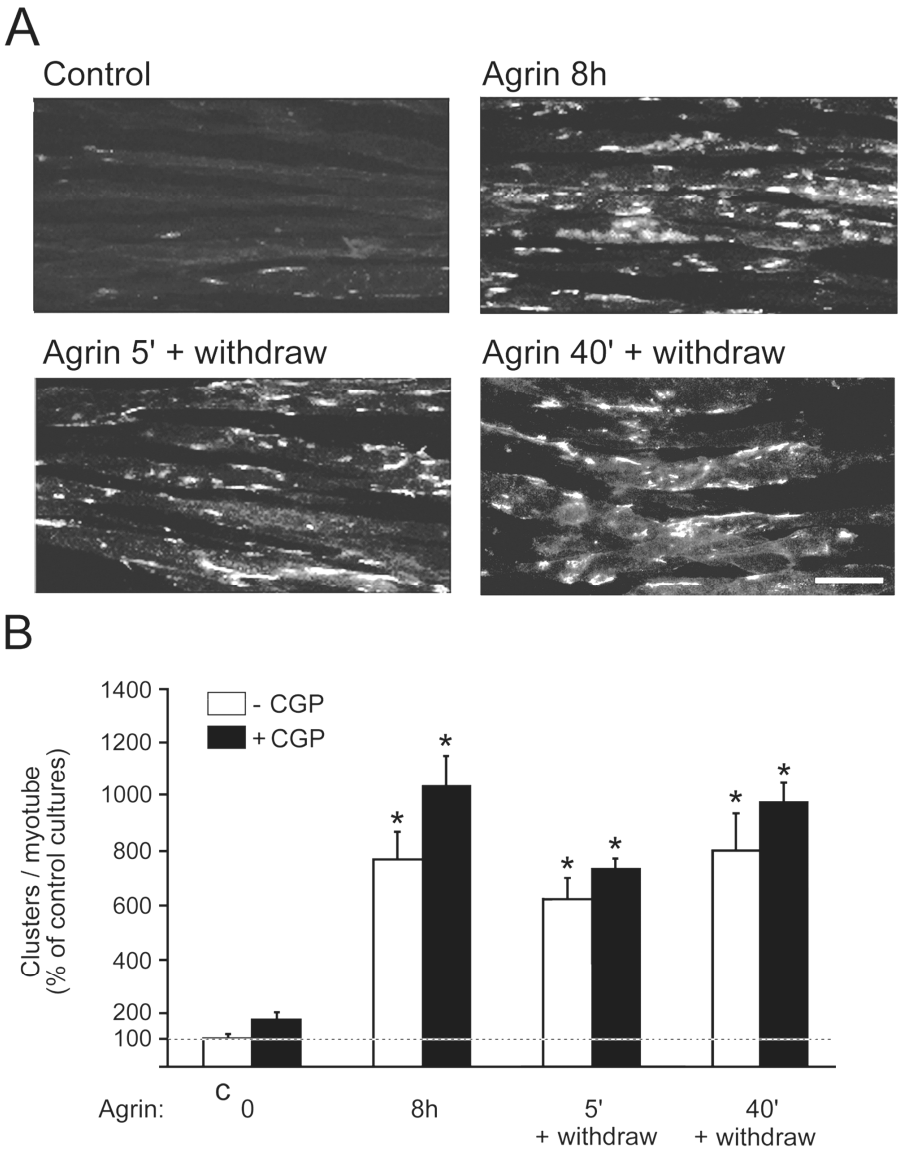


**Figure 2.7. A single 5 min agrin pulse leads to efficient AChR clustering that is not affected by CGP77675.** In the presence or absence of CGP77675, C2 myotubes were treated for 8 h continuously with 0.5 nM agrin, or for 5 or 40 min followed by withdrawal of agrin for 8 h. AChRs were stained with rhodamine-α-BTX and analysed by fluorescence microscopy. Scale bar, 40 µm. (B) The numbers of AChR clusters per myotube are shown as % of untreated cells (c, control; mean ± SEM of at least 5 experiments). * Differ significantly from control ($p<0.01$), but not from each other ($p>0.09$, by two-tailed paired t-test).

We next directly visualised, by immunoblotting, agrin that remained attached to myotubes following a brief treatment and withdrawal. The immunoblot assay was very sensitive: by densitometric scanning and comparison to an agrin standard of known concentration, we calculated that the detection limit of agrin in the immunoblot is 2.4 femtomole / mg cellular protein. While cell-bound agrin was readily detectable (300 fmole/mg) after a continuous 8 h incubation with 0.5 nM agrin (Figure 2.8A, lane 6), cell-bound agrin was not detectable after a 5 min pulse followed by withdrawal (Figure 2.8A, lanes 2 and 3). Following a 40 min pulse and a 40 min withdrawal period, low levels of agrin were detectable; following a longer withdrawal period (8 h), myotube-associated agrin was no longer detectable (Figure 2.8A, lanes 4 and 5). As the extent of AChR clustering is the same in myotubes treated with agrin for 5 min, followed by an 8 h withdrawal, and in myotubes treated continuously with agrin for 8 h (Figure 2.7), the extent of AChR clustering is not correlated with the amount of agrin remaining associated with the myotubes after the pulse (Figure 2.8A). Thus the remaining agrin – if present at all – is not critical for clustering. Rather, the brief stimulation with agrin appears sufficient to trigger a response that occurs several hours later in the absence of agrin.

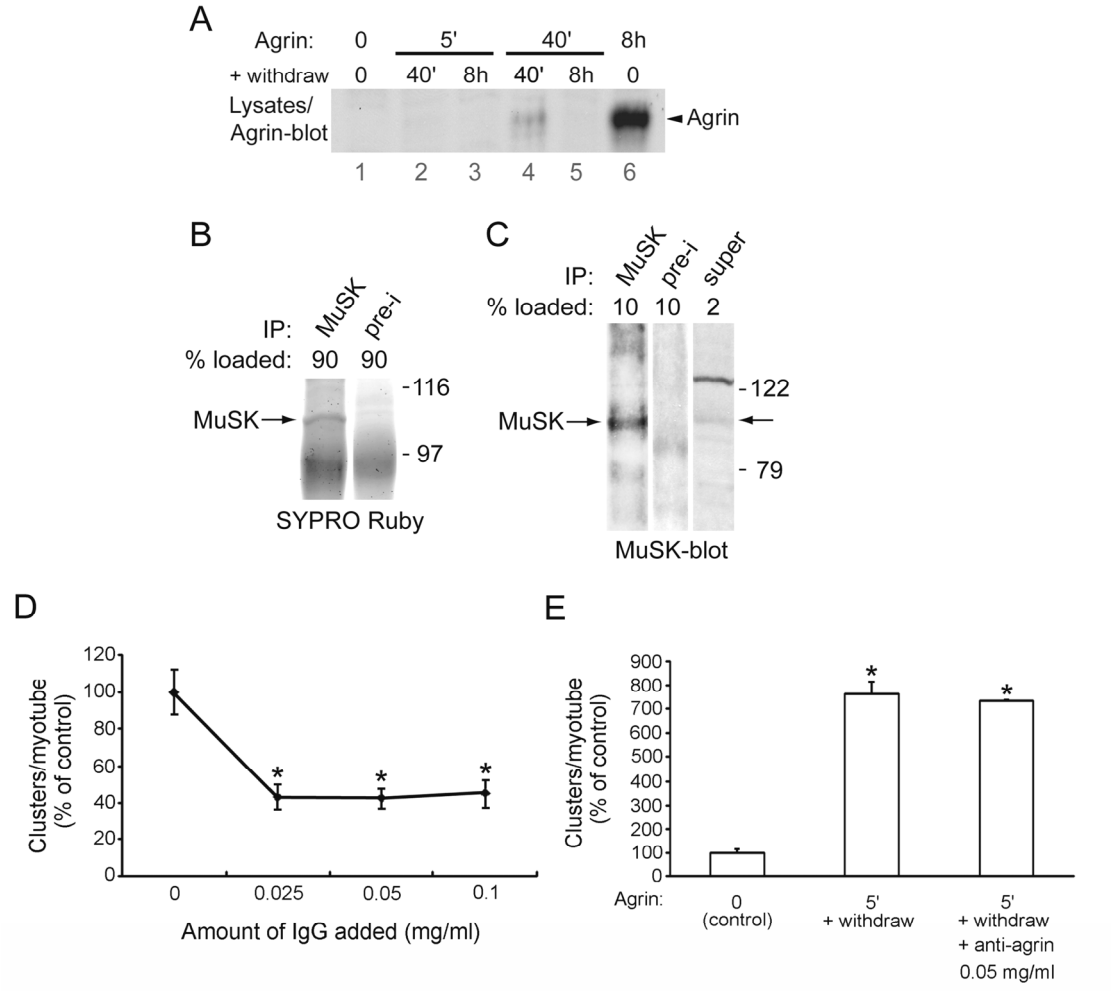


**Figure 2.8. Little, if any agrin remains cell-bound after a brief agrin pulse and is not responsible for clustering.** (A) C2 myotubes were pulse-treated with agrin (0.5 nM for 5 or 40 min), followed by washing and withdrawal for 40 min or 8 h; parallel samples were treated continuously with agrin for 8 h. Cell lysates were analysed by agrin-immunoblotting. No agrin-signal of ca.120 kDa (representing recombinant agrin, C-$Ag_{12,4,8}$), is detectable in cells treated for 5 or 40 min followed by 8 h withdrawal (lanes 3 and 5). A small agrin-signal is visible in cells treated for 40 min followed by 40 min withdrawal (lane 4), but this does not correlate with clustering, which is the same after a 5 min or 40 min pulse (see Figure 2.7). (B) MuSK was immunoprecipitated from C2 lysates and 90% of the precipitate was analysed by SDS-PAGE and SYPRO Ruby protein staining. MuSK was identified based on its molecular weight and its absence in pre-immune control precipitation. Using a parallel BSA standard (not shown), the amount of precipitated MuSK was determined in the gel (100 ng). (C) From the same samples as in B, 10% of the MuSK-precipitate and 2% of the supernatant were analysed by MuSK-immunoblotting. By densitometric scanning, the efficiency of the immunoprecipitation was calculated (60%). (D) In the presence or absence of different concentrations of anti-agrin antibody mAb33, C2 myotubes were treated for 6 h with agrin, and AChR clustering quantified as described for Figure 2.5. * Differs significantly from control (no IgG; $p<0.001$, by two-tailed paired t-test). (E) Myotubes were treated with 0.5 nM agrin for 5 min, followed by withdrawal of agrin, washing and incubation in agrin-free medium in the presence or absence of 0.05 mg/ml of mAb 33. After 8 h, AChRs were stained with rhodamine-α-BTX and quantitated as in Figure 2.5. * Differs significantly from control ($p<0.001$), but not from each other ($p>0.09$, by two-tailed paired t-test).

To rule out that MuSK-bound agrin may remain on MuSK after withdrawal but represent only a small portion of myotube-associated agrin not detectable by our assay, we used an indirect method to measure the level of MuSK protein. We first precipitated MuSK from myotube lysates using bead-coupled MuSK-antibodies (Figure 2.8B). The amount of this precipitated MuSK was estimated using a SYPRO Ruby protein stain and a parallel BSA standard. Second, the efficiency of this precipitation was calculated by a parallel MuSK-Western blot, comparing precipitated with remaining soluble MuSK (Figure 2.8C). These estimations yielded an amount of 179.1 femtomole MuSK / mg cellular protein (see Materials and Methods for details). This value is likely to be an underestimation, since due to the background in the SYPRO Ruby stain we probably underestimate the amount of precipitated MuSK. Nonetheless, our estimation demonstrates that in myotubes the level of MuSK (179.1 fmole/mg) is much higher than the level of agrin remaining cell-bound after withdrawal (<2.4 fmole/mg). Since our recombinant agrin (C-terminal half of agrin; C-$Ag_{12,4,8}$) (Ferns et al., 1996) not only binds to the MuSK complex but also to heparin and α-dystroglycan (Gesemann et al., 1996), the actual level of agrin remaining MuSK-bound may be substantially less than 2.4 fmole/mg. Taken together, these data strongly suggest that after withdrawal the vast majority of agrin was released from myotubes and from MuSK on these myotubes.

Finally, we analysed the effect of an anti-agrin antibody, mAb33 (Hoch et al., 1994) on AChR clustering. When agrin was incubated with the antibody and added to myotubes, clustering of AChRs was severely reduced, as reported previously (Hoch et al., 1994) (Figure 2.8B). In contrast, mAb33 did not inhibit AChR clustering when added to myotubes in the withdrawal period immediately after a 5 min pulse of agrin (Figure 2.8C). Although we cannot exclude the possibility that myotube-associated agrin is inaccessible to the antibody, these data suggest that agrin, which may be bound to myotubes after the pulse, is no longer required for AChR clustering.

Thus, many lines of evidence indicate that the withdrawal of agrin was effective. First, our agrin, C-$Ag_{12,4,8}$, lacks the N-terminal domain of agrin, which is required for efficient binding to the extracellular matrix (Denzer et al., 1995). The interaction of C-$Ag_{12,4,8}$ with myotubes is therefore rather weak, allowing this agrin to be washed

off easily. Second, consistent with this notion, no detectable agrin remained associated with myotubes after a 5 min agrin pulse followed by withdrawal and washing. If some agrin remained, it did not appear critical for clustering and was present at much lower amounts than MuSK. This implies that agrin was efficiently released from MuSK, although we cannot rule out that a very small amount of agrin (ca. 1% of MuSK) remained MuSK-associated. Third, no agrin activity was detectable in the medium following withdrawal. Fourth, a blocking antibody to agrin, added during the withdrawal phase, failed to inhibit AChR clustering initiated by a 5 min pulse of agrin. Taken together, these experiments show that agrin acts rapidly, to trigger a clustering mechanism that subsequently acts autonomously in the absence of further agrin stimulation.

### *2.4.8 MuSK phosphorylation increases rapidly after the agrin pulse and leads to maximal AChR clustering in the absence of agrin*

Since agrin acts so rapidly, we examined the level of MuSK tyrosine phosphorylation at the end of the agrin pulse and during the withdrawal period. At 0.5 nM agrin, strong MuSK phosphorylation was seen after 5 or 40 min (Figure 2.9A). Following 8 h withdrawal, these levels of MuSK phosphorylation led to a similar number of AChR clusters (Figure 2.9B), which were indistinguishable from a continuous 8 h agrin treatment (Figure 2.7). As expected, the rate of MuSK phosphorylation was slower when myotubes were stimulated with 0.1 nM agrin. Consequently, the level of MuSK phosphorylation was much less at 5 min in myotubes treated with 0.1 nM agrin than in myotubes treated with 0.5 nM agrin, but similar at 40 min with both agrin concentrations (Figure 2.9A). The extent of clustering paralleled these phosphorylations: after a 5 min pulse, clustering was less efficient at 0.1 than at 0.5 nM agrin, while after a 40 min pulse, clustering was comparable at both agrin concentrations (Figure 2.9B). This implies that a certain critical level of MuSK activation must be reached within or after the agrin pulse in order to achieve maximal clustering: 5 min of 0.1 nM agrin does not reach this level while 5 min of 0.5 nM agrin reaches it.

After 5 min of agrin treatment, MuSK phosphorylation increases significantly in the continued presence of agrin, to reach a peak after 40 min (Fuhrer et al., 1997). We analysed MuSK phosphorylation after the 5 min agrin pulse, particularly addressing whether this phosphorylation is further increased during the withdrawal period. At both 0.5 and 0.1 nM of agrin, MuSK phosphorylation indeed increased substantially after the pulse within 10-35 min (Figure 2.9C). In comparison, continuous incubation with agrin for 40 min led to a still higher level of MuSK phosphorylation at 40 min (Figure 2.9C). After 8 h, the continuous and pulsed agrin treatments resulted in identical MuSK phosphorylation (see Figure 2.6). These data demonstrate that MuSK phosphorylation increases rapidly following a brief agrin pulse, even though cell-bound agrin is not detectable during this increase. Thus, agrin triggers a mechanism that increases MuSK phosphorylation in the subsequent absence of agrin. If MuSK phosphorylation reaches a certain critical level, as is the case using a 5 min 0.5 nM agrin pulse, maximal AChR clustering is seen after 8 h of withdrawal. If the MuSK phosphorylation is below this level, as in the case of a 5 min 0.1 nM agrin pulse, maximal clustering does not occur (Figure 2.9A), even though MuSK phosphorylation still increases following withdrawal. This defines a critical level of MuSK activation that can be reached within minutes, beyond which the maximal AChR clustering program is initiated even in the absence of further agrin stimulation.

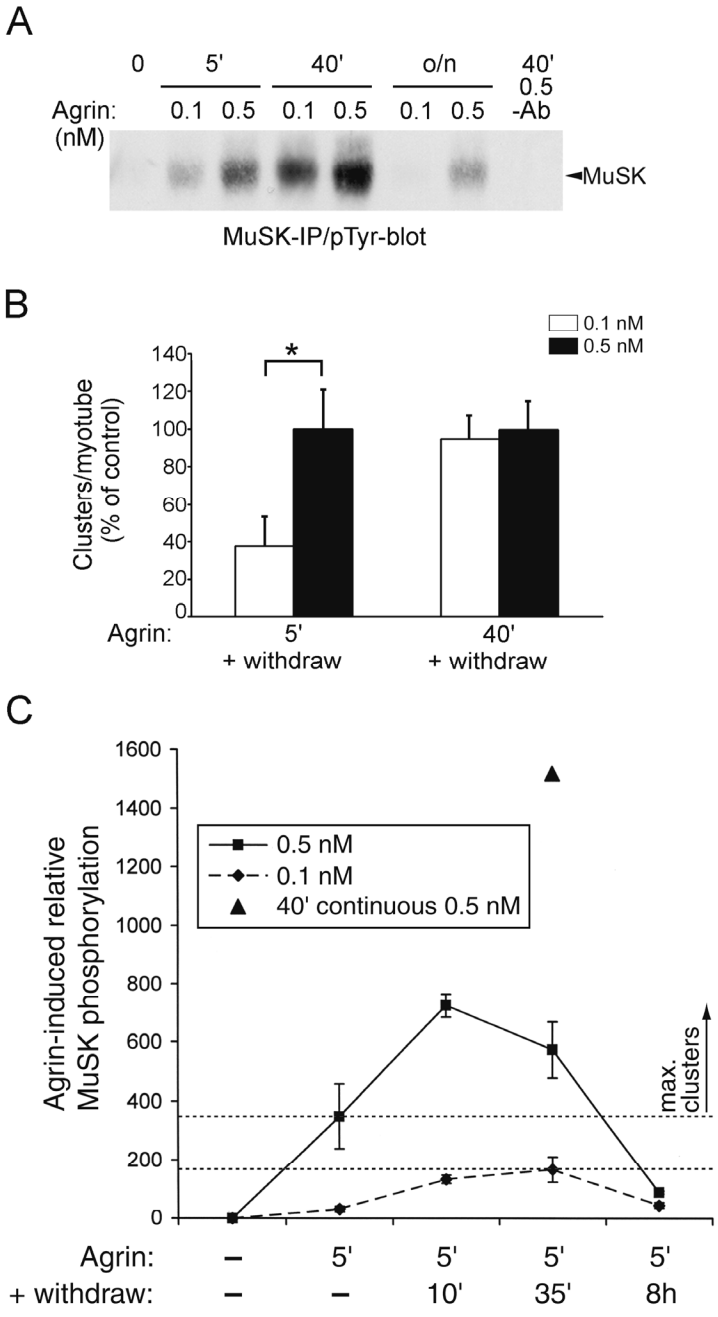


**Figure 2.9. The agrin-triggered autonomous pathway is maximally activated if MuSK phosphorylation reaches a certain level.** (A) C2 myotubes were treated 5 min, 40 min or overnight continuously with 0.1 or 0.5 nM agrin, and cell lysates were analysed by MuSK immunoprecipitation and phosphotyrosine immunoblotting. As control, MuSK-antibody was omitted (-Ab). (B) C2 myotubes were treated for 5 or 40 min with 0.1 nM or 0.5 nM agrin, followed by withdrawal for 8h. AChRs were stained with rhodamine-α-BTX and analysed by fluorescence microscopy. Within each pulse, the numbers of AChR clusters per myotube treated with 0.5 nM agrin, quantitated as in Figure 2.5, were set to 100 %. *$p<0.02$, by two-tailed paired t-test. (C) Myotubes were treated for 5 min with 0.1 nM (diamonds) or 0.5 nM (squares) agrin, followed by 10 min, 35 min or 8 h of agrin withdrawal, MuSK immunoprecipitation and phosphotyrosine immunoblotting. Parallel cells were treated for 40 min continuously with 0.5 nM agrin (triangle). After a 5 min agrin pulse, MuSK phosphorylation increases in the withdrawal period. Quantitation shows the mean ± SEM of 3 experiments evaluated by densitometric scanning. The area between the drawn lines indicates a critical level of MuSK phosphorylation. Above this level, maximal clustering occurs even in the subsequent absence of agrin.

## 2.5 Discussion

Here we reveal novel temporal patterns of tyrosine kinase action in the agrin signalling pathway. First, we show that SFKs are required for phosphorylation of MuSK and AChRs early (5 min) but not later (40 min) after agrin stimulation, whereas Abl kinases mediate late phosphorylation. Second, we find that SFKs act during the period of AChR cluster formation, and not thereafter, to stabilize AChR clusters following withdrawal of agrin. Thus, during cluster initiation, SFKs recruit a stabilisation pathway that acts hours later. Third, we find that a single, brief pulse of agrin is sufficient to induce a pathway that clusters AChRs efficiently in the absence of agrin. This autonomous pathway, once triggered, leads to a rapid increase in MuSK phosphorylation; when a critical level of MuSK phosphorylation is reached, the clustering program is fully activated, leading to AChR clusters hours later.

### *2.5.1 SFK activity is dispensable for agrin-induced AChR cluster formation*

Our data clarify the role of SFKs in the formation of AChR clusters. Previous studies yielded differing results (Mohamed et al., 2001; Smith et al., 2001), which may be attributed to inadvertent inhibition of Abl by the inhibitor PP1 (Tatton et al., 2003). As Abl is required for AChR clustering (Finn et al., 2003), high concentrations (20 μM) of PP1 may have interfered with Abl, leading to defective AChR clustering (Mohamed et al., 2001), whereas lower concentrations of PP1 (5 μM) may have been more selective for SFKs, leading to normal clustering (Smith et al., 2001). Our findings, combining gene ablation with more selective pharmacological inhibition, now demonstrate that the formation of clusters requires very little if any SFK activity: in wild-type and even *src*$^{-/-}$*;fyn*$^{-/-}$ cells, agrin induces normal AChR clustering in the presence of CGP77675, a potent and specific inhibitor that efficiently blocks SFKs including Yes (Missbach et al., 1999). Because Yes is the main remaining SFK in

*src*$^{-/-}$;*fyn*$^{-/-}$ cells (Smith et al., 2001), these data provide strong evidence that SFK activity is not needed for AChR cluster formation.

### *2.5.2 Src-family and Abl kinases mediate early and late phosphorylation, respectively*

In agrin-induced phosphorylation of AChR β and δ subunits, the role of SFKs has also remained controversial (Mohamed et al., 2001; Smith et al., 2001). We now find that SFK activity acts only early (5 min) after addition of agrin. These findings may provide an explanation for the reported failure of PP1 to reduce AChR β phosphorylation following 30-40 min of agrin stimulation (Smith et al., 2001) and inhibition of AChR β phosphorylation by PP1 following a 10 min treatment with agrin (Mohamed et al., 2001). Thus, our results establish a temporal pattern of SFK action in agrin signalling in which these kinases are required for MuSK and AChR phosphorylation only early (5 min) but not later (40 min and thereafter). In contrast, Abl kinases cause MuSK and AChR β phosphorylation preferably late (40 min) in agrin signalling. Phosphorylation of MuSK and AChRs is thus under complex temporal control and involves several non-receptor tyrosine kinases.

### *2.5.3 Time-delayed action of SFKs in stabilisation of AChR clusters*

We find that SFK activity is required selectively during agrin-induced AChR cluster formation, to stabilize these clusters in the hours after agrin withdrawal. These observations functionally link the SFK activation during AChR cluster formation to cluster stabilisation later. Since SFK activation is maximal after 40 min of agrin treatment (Mittaud et al., 2001) (see also Figure 2.1), i.e. hours before AChR clusters are formed, our data imply that SFKs already recruit a stabilisation pathway when AChR clustering has just been initiated.

As MuSK and AChR phosphorylation occur independently of SFKs after 40 min of agrin treatment, these data suggest that SFKs phosphorylate proteins in addition to

MuSK and AChRs, which are crucial for AChR cluster stabilisation. This is in good agreement with our observation that SFKs are not activated by C-terminal dephosphorylation (Figure 2.1) but, by inference, through protein interactions (Thomas and Brugge, 1997), implying the existence of other SFK-binding proteins and SFK-substrates. Agrin-induced SFK activity is thus under constant control rather than being maximal and sustained over prolongated periods (Thomas and Brugge, 1997). Of interest is the reduction in phosphorylation of $Y_{215}$ within the SH2 domain of SFKs (Figure 2.1), a domain known to mediate protein interactions, for example with phosphorylated MuSK (Mohamed et al., 2001). Dephosphorylation of $Y_{215}$ may well render the SH2 domain capable of binding to further proteins. A good candidate is α-dystrobrevin, whose long isoform (α-dystrobrevin-1) mediates AChR cluster stabilisation in part due to its C-terminal tyrosine phosphorylation sites (Grady et al., 2003), which may be SFK substrates. Other candidates are the Src-substrates talin, paxillin, α-fodrin and cortactin, which are all enriched at NMJs and AChR clusters in myotubes (Dai et al., 2000; Hall and Sanes, 1993). All these proteins interact with actin, whose polymerization is a requirement for AChR clustering and may stabilize clusters (Dai et al., 2000).

The link between cluster formation and their stabilisation by SFKs may involve differential phosphorylation of MuSK mediated by SFKs early (5 min) and by Abl kinases late (40 min) in agrin signalling. Although Abl kinases can be phosphorylated by SFKs (Tanis et al., 2003), our observed effects of CGP77675 and STI 571 show that Abl acts independently of SFKs at 40 min, and that SFKs act independently of Abl at 5 min to phosphorylate MuSK. Nevertheless the action of the two kinases is highly intertwined, since early-acting SFKs lead to a delayed pathway that stabilizes AChRs clusters, while late-acting Abl kinases lead to a more immediate pathway that forms clusters. This may well occur through phosphorylation of separate tyrosine residues in MuSK by Abl and SFKs. The juxtamembrane domain tyrosine ($Y_{553}$) is a good candidate for Abl, since both $Y_{553}$ and Abl are required for AChR cluster formation (Finn et al., 2003; Herbst and Burden, 2000; Zhou et al., 1999). Tyrosines in the N- and C-terminal region of the MuSK kinase domain ($Y_{576}$ and $Y_{812}$, respectively) are candidates for SFKs, since they are not essential in the formation of clusters (Herbst and Burden, 2000), but may still play a role in their stabilisation.

Phosphotyrosines may bind to specific adaptor proteins, leading to activation of distinct postsynaptic pathways. These pathways may be initiated and maintained through the increased association of both Abl and SFKs with MuSK, which is rapidly triggered by agrin (Finn et al., 2003; Mohamed et al., 2001).

Taken together, the emerging picture is that a complex containing MuSK, Abl and SFKs is rapidly assembled by agrin. Abl and SFKs, possibly through phosphorylation of distinct MuSK residues, activate pathways for cluster formation and stabilisation, respectively. Besides with MuSK (Mohamed et al., 2001), SFKs interact with AChRs (Fuhrer and Hall, 1996), and agrin stimulates association between MuSK and AChRs (Fuhrer et al., 1999; Fuhrer et al., 1997). Therefore, the stabilisation program triggered by SFKs during initiation of clustering is recruited to the right location, nascent clusters at postsynaptic membrane specialisations containing MuSK and AChRs. The delay in its manifestation may be important to ensure that the pathway becomes effective only after the initial clusters have been formed, to achieve cluster stabilisation and maturation. This delay may thereby be related to the complex elaboration of the NMJ, including the formation of postjunctional folds and restriction of AChRs to the crests of these folds, which occur postnatally. Thus, complex interactions between MuSK, Abl, SFKs and AChRs may ensure that pathways for cluster formation and stabilisation/maturation converge in an appropriate manner both temporally and spatially at the nascent NMJ.

### *2.5.4 A single brief agrin pulse activates an autonomous mechanism that leads to AChR clustering*

Our data reveal another temporal aspect of tyrosine kinase action in agrin signalling, because the continuous stimulation by agrin is not required for AChR clustering. Instead, using an agrin pulse and withdrawal protocol, we find that agrin initiates rapidly, within a single 5 min pulse, a clustering pathway that then acts autonomously in agrin's absence.

For other growth or differentiation factors, rapid activation of their tyrosine kinase receptors has been intensively investigated and occurs widely (Weiss and

Schlessinger, 1998). It remains largely unknown, however, to what extent the continual presence of ligand is necessary, after the initial trigger, to maintain the activity of intracellular signalling. In neuregulin signalling in myogenesis, which requires several days, and in basic FGF-induced calcium influx in fibroblasts, the continuous presence of ligand seems necessary to achieve prolongated effects (Florini et al., 1996; Munaron et al., 1995). In contrast, using an approach similar to ours, a single brief pulse of NGF, followed by withdrawal and inhibitory antibodies, was exploited to show that NGF acts rapidly to trigger certain long-lasting pathways in PC12 cells (Toledo-Aral et al., 1995). The brief application of NGF, EGF or basic FGF induced sodium channel gene expression hours later, leading to global cell excitability (Toledo-Aral et al., 1995).

Agrin induces phosphorylation of MuSK within 5 min, but this phosphorylation reaches a peak only after 40 min (Fuhrer et al., 1997). We show here that continuous stimulation by agrin is not necessary. In comparison to other receptor tyrosine kinases, MuSK is very unusual and its tasks are far more complex, as previously noted by others (Glass et al., 1997): MuSK not only triggers intracellular signalling but has to localise such signalling to the correct site of a huge syncytial cell, to achieve new protein synthesis as well as relocalization of dozens of pre-existing proteins at the nascent postsynapse. In addition, MuSK is required for presynaptic specialisation of the nerve terminal (Glass et al., 1996). In line with such complex roles of MuSK, experiments with chimaeric receptors have shown that, in contrast to growth factor receptors, MuSK's activated intracellular domain alone is not sufficient to reproduce MuSK's biological activity (i.e., AChR clustering) – structural portions of the MuSK extracellular domain are also important (Glass et al., 1997). Our data propose that even responses as complex as those initiated by MuSK, involving structural roles as well as catalytic tasks, may be triggered by a single brief pulse of ligand. This opens new avenues to better understand signalling by complex receptor tyrosine kinases in general.

Our data show that within this rapidly triggered, autonomous pathway of agrin, the phosphorylation level of MuSK is of central importance. We find that it increases rapidly even after agrin withdrawal and, when reaching a certain critical level within 40 min of withdrawal, leads to maximal AChR clustering many hours later. This

increase in MuSK phosphorylation may originate from MuSK itself through autophosphorylation, or from an associated kinase. As mentioned above, MuSK occurs in a multiprotein complex where it associates at least with SFKs (Mohamed et al., 2001), Abl kinases (Finn et al., 2003), some AChRs (Fuhrer et al., 1999; Fuhrer et al., 1997), dishevelled (Luo et al., 2002), geranylgeranyltransferase (Luo et al., 2003b) and indirectly with rapsyn (Apel et al., 1997). We recently found MuSK in association with the tyrosine phosphatase SHP-2 in cultured myotubes (R. Willmann, A. Camilleri, C. Fuhrer, unpublished observations). MAGI-1c is another protein that at least in Torpedo electric organ associates with MuSK (Strochlic et al., 2001). The plethora of MuSK-associated partners opens the possibility that agrin binding to its receptor complex may rapidly trigger conformational changes in such associated proteins in combination with kinase activation (Abl, SFKs) and/or phosphatase inactivation (possibly SHP-2). Such mechanisms could hold MuSK in an activated state independently of bound agrin and increase its phosphorylation status. In agreement with this, ectopic overexpression in muscle of constitutively active MuSK induces AChR aggregation in the absence of agrin (Jones et al., 1999), showing that MuSK can cause AChR clustering independently of agrin.

Our mechanism of MuSK activation proposes that when a critical level of MuSK phosphorylation is reached, the maximal clustering program is initiated. This mechanism may explain two prominent aspects of AChR clustering at the NMJ. The first is that myotubes contain several inhibitory mechanisms that counteract clustering. Rapsyn, for example, does not form clusters ectopically in mammalian muscle in vivo (Marangi et al., 2001) and its clustering is marginal in cultured myotubes not treated with agrin, although rapsyn associates with AChRs in such cells (Moransard et al., 2003). In contrast, rapsyn alone efficiently self-aggregates in heterologous cells (Froehner et al., 1990; Phillips et al., 1991). This implies that muscle-specific inhibition prevents rapsyn from clustering unless agrin and a synaptic environment are present. Another inhibitory mechanism are tyrosine phosphatases that dephosphorylate the AChR constitutively as shown by a strong increase in receptor phosphorylation by pervanadate treatment in the absence of agrin (Mohamed et al., 2001; Wallace, 1995). Thus the tyrosine kinase cascade downstream of MuSK is continuously counteracted by phosphatases which must be overcome by kinase

activation through agrin (Wallace, 1995). Our data imply that agrin overrides the inhibition associated with rapsyn and phosphatases in a threshold-like mechanism: when a critical level of MuSK phosphorylation is reached after a single agrin pulse, inhibition is overcome allowing clustering to proceed autonomously and maximally. Besides recruitment of downstream signalling partners to critical phosphotyrosines in MuSK (see above), this may involve MuSK-mediated phosphorylation of downstream effectors that may operate autonomously beyond a certain level of their activation.

The second feature of NMJ development for which our mechanism of MuSK activation may be important is the highly regulated spatial AChR distribution in which high receptor densities lie only microns apart from regions that contain very few AChRs (Hall and Sanes, 1993; Sanes and Lichtman, 2001). Signalling underlying clustering must therefore operate with a high spatial resolution. This could be achieved by a mechanism in which agrin stimulation, when producing a critical level of MuSK phosphorylation, triggers maximal clustering autonomously. In regions where stimulation is beyond this level, clustering would thereby occur maximally, while in regions where this level is not reached, the inhibitory mechanisms detailed above would strongly reduce cluster formation. Thereby the postsynaptic membrane may be sculpted in vivo, and the effector proteins in such a cascade will be interesting topics of future research.

### *2.5.5 A rapidly triggered autonomous agrin pathway – a mechanism in the central nervous system?*

In contrast to neurotransmitter receptors at synapses in the central nervous system (CNS), AChR clustering at the NMJ occurs much slower. CNS receptors can cluster and synapses form within 1-2 h (Cohen-Cory, 2002). Increasing evidence suggests that agrin is found concentrated at interneuronal synapses (Hoover et al., 2003; Koulen et al., 1999) and may also play a role in synapse formation, including receptor clustering, between neurones (Bose et al., 2000; Ferreira, 1999; Gingras et al., 2002), besides its importance at the immunological synapse (Khan et al., 2001). So how could agrin act in the CNS if it takes so long to cluster muscle AChRs? The features

revealed here show that agrin activates its receptor and initiates the AChR clustering pathway extremely rapidly. Thus, it is not the agrin itself that is slow in activation the agrin receptor, but the downstream signalling pathway in muscle is slow in ultimately leading to AChR clusters. If coupled to a more rapid downstream pathway in neurones, a CNS agrin receptor – for which there are indications (Hoover et al., 2003) – would therefore be in a position to rapidly form a synapse. The rapid action of agrin as inferred from our study could thereby rapidly lead to CNS receptor clustering, rapid enough for a CNS synapse to form.

Studies on the NMJ have shown that AChR clustering is under complex control, involving positive as well as negative regulation. Laminin converges with agrin signalling at the level of rapsyn (Marangi et al., 2002), while neurotrophins (BDNF and NT-4) and neuregulin can inhibit AChR clustering (Trinidad and Cohen, 2004; Wells et al., 1999). Neurones may in an analogous way have multiple pathways that affect neurotransmitter receptor clustering. While an autonomous pathway rapidly triggered by agrin may be one mechanism for induction of CNS synapses, CNS synapse formation may be influenced by a more complex network of positive and negative signals that reflects the dynamic nature of synapses in the brain.

## 2.6 Acknowledgements

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# Chapter 3

## *A balance between tyrosine phosphatases and Src-family kinases stabilises clusters of acetylcholine receptors*

This chapter represents a manuscript in preparation, which will soon be submitted (Alain A. Camilleri, Matthias Gesemann, and Christian Fuhrer, manuscript in preparation).

## 3.1 Abstract

Protein tyrosine phosphorylation plays a major role in most cellular signalling mechanisms. The interplay between protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) allows for high spatial and temporal regulation of phosphorylation events. At the neuromuscular synapse, phosphorylation of muscle-specific kinase MuSK is triggered by agrin released from the nerve terminal, and leads to phosphorylation, clustering and stabilisation of postsynaptic acetylcholine receptors (AChRs). Src-family kinases (SFKs) and Abl kinases are two families of PTKs known to be important in these events, but little is known about the role of PTPs. We have used pharmacological inhibition by sodium pervanadate to investigate the role of PTPs in the agrin-induced clustering and stabilisation of AChRs. We report that phosphatase inhibition interferes with agrin-induced clustering, and renders these clusters unstable following agrin removal. This pathway appears to involve the participation of SFKs, since in their absence in *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes or in wild-type myotubes in which SFKs have been blocked using PP2, the inhibition of PTPs rescues the stability of AChR clusters. We also show that pervanadate treatment decreases Src activity in wild-type cells, important in stability of AChR clusters. We identify SHP-2 as a candidate PTP in this pathway. SHP-2 associates increasingly with MuSK upon stimulation by agrin in myotubes, and colocalises with MuSK in heterologous cells. Knocking down SHP-2 in myotubes leads to a reduction in AChR clusters, and increased instability of agrin-induced clusters following agrin withdrawal. Increased levels of SHP-2 in *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes could contribute to the instability seen in AChR clusters in these cells. These data reveal that a tight balance between PTK and PTP activities in muscles is required during development to allow for an agrin-triggered signalling cascade to lead to proper clustering and stabilisation of AChR clusters at the NMJ.

## 3.2 Introduction

The neuromuscular junction (NMJ) offers an easily accessible model at which to study the process of synaptogenesis. The initial steps in the formation of this specialised synapse involve changes on both the presynaptic nerve terminal and the postsynaptic muscle membrane. Important signalling events in the various phases of development of the adult synapse involve tyrosine phosphorylation and dephosphorylation events. This implies an interplay between tyrosine kinases and phosphatases whose net activities regulate these signalling processes. Signalling events in and below the postsynaptic muscle membrane are initiated early on by the release of agrin from the presynaptic nerve terminal. This causes rapid phosphorylation of the muscle-specific kinase (MuSK) on the muscle surface (Hopf and Hoch, 1998). Subsequent downstream steps lead to the clustering of acetylcholine receptors (AChRs) beneath the nerve terminal at a very high density (10-20,000 per $\mu m^2$) (Sanes and Lichtman, 1999). Besides agrin and MuSK (DeChiara et al., 1996; Gautam et al., 1996), this process requires the presence the 43 kDa protein rapsyn (Gautam et al., 1995), which associates with and anchors the AChR (Moransard et al., 2003). The high density of AChRs allows for normal transmission of the signal between nerve and muscle to evoke muscle contraction. AChR clusters become stably anchored to the cytoskeleton, allowing the neuromuscular synapse to last through adulthood. The signalling events leading to the clustering process, as well as those involved in the stabilisation of the AChR clusters have not been fully elucidated. However several important tyrosine kinase players have been discovered over the past decade. These include Abl (Finn et al., 2003) and Src-family kinases (SFKs) (Mohamed et al., 2001), both important in mediating agrin-induced phosphorylation of MuSK and the AChR β subunit (Mittaud et al., 2004). AChR β phosphorylation is also important in stabilising the receptor by linking it to the actin cytoskeleton, a process which requires SFKs (Mohamed et al., 2001; Sadasivam et al., 2005). SFKs are however most important in the stabilisation on AChR clusters. *In vivo*, electroporation of dominant-negative Src disrupts the postsynaptic apparatus

(Sadasivam et al., 2005), and in cultured *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes, AChR clusters disintegrate rapidly after removal of agrin from the medium (Smith et al., 2001).

The importance of protein tyrosine phosphatases (PTPs) in the control and modulation of the tyrosine phosphorylation-induced signalling events is unquestionable (Wallace, 1995). However new studies on their role in the signalling networks for the development of the neuromuscular synapse have only recently re-emerged. The inhibition of PTPs using sodium pervanadate has shown their involvement in controlling the phosphorylation status of AChR β, γ and δ subunits, the link of AChRs to the cytoskeleton, and mobility of the receptors in the muscle membrane; hence the requirement of PTPs in agrin-induced AChR clustering (Meier et al., 1995; Wallace, 1995). PTPs were also shown to control MuSK phosphorylation by agrin, and in restricting the AChR (Madhavan et al., 2005) and phosphotyrosine cluster boundary beneath growth-factor coated beads in muscle cells in culture (Dai and Peng, 1998). The non-receptor PTP SHP-2 has emerged as a possible candidate phosphatase playing a key role in signalling events at the neuromuscular junction (NMJ) (Madhavan et al., 2005; Meier et al., 1995; Tanowitz et al., 1999). SHP-2 was implied in AChR clustering and in controlling AChR gene expression induced by neuregulin (Madhavan et al., 2005; Tanowitz et al., 1999).

The role played by PTPs in agrin-induced AChR clustering and in the subsequent stability of these clusters is investigated. Our data show the importance of a balance between SFK activity and PTP activity in both *src*$^{-/-}$;*fyn*$^{-/-}$ and wild-type myotubes. However, whilst PTPs stabilise AChR clusters in wild-type cells, they destabilise the clusters in the absence of Src and Fyn activity. The role of phosphatase activity on the phosphorylation status of AChRs, MuSK and SFKs is also studied. SHP-2 is shown to associate increasingly with MuSK in myotubes when MuSK is phosphorylated by agrin, and colocalises with MuSK in heterologous cells. The functional role of SHP-2 as a candidate PTP with activity during neuromuscular synapse development is addressed using shRNA to knockdown SHP-2 in myotubes, showing the importance of PTPs and SHP-2 in controlling maintenance of AChR stability in muscles.

## 3.3 Materials and Methods

### *3.3.1 Expression of agrin and cell culture*

Soluble neural agrin (C-$Ag_{12,4,8}$) was produced in COS cells as previously described (Fuhrer et al., 1997). Reagents for cell culture were purchased from Invitrogen AG (Basel, Switzerland). C2C12 (C2) mouse muscle cells were propagated as myoblasts on 3.5 or 6 cm (Corning), and 10 or 15 cm plastic dishes (Nunc) in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l D-glucose and pyruvate, supplemented with 20% foetal bovine serum, 0.5% chick embryo extract, 2 mM glutamine and penicillin/streptomycin. After reaching 90-100% confluence, cells were shifted to C2 fusion medium containing DMEM, 5% horse serum and 2 mM glutamine and penicillin/streptomycin. Fusion of myoblasts to generate myotubes was evident after 1 day. By 2-3 days, contracting myotubes were usually observed, and cells were used for experiments (Fuhrer et al., 1997). *src*$^{-/-}$;*fyn*$^{-/-}$ myoblasts (clone DM15), and the corresponding wild-type myoblasts (SW10) were grown in 6 and 10 cm plastic plates (Nunc) in DM growth medium and switched to DM fusion medium to form myotubes as previously described (Sadasivam et al., 2005; Smith et al., 2001).

### *3.3.2 Inhibitors*

Sodium pervanadate was prepared as previously described (Megeath et al., 2003; Wallace, 1995). One part of 500 mM hydrogen peroxide was added to 50 parts of 10 mM sodium orthovanadate (Sigma) (pre-boiled at 100°C for 10 minutes) in modified Tyrodes solution (145 mM NaCl, 5 mM KCl, 5.5 mM glucose, 40 μM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES pH 7.4). The mixture was shaken for 10 min at room temperature and diluted in fusion medium to defined concentrations immediately before use. Cultures were routinely treated with 20 μM pervanadate. Src-class kinase inhibitor PP2 [4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine]

(Calbiochem) was diluted into cell culture medium to a final concentration of 10 μM (Charpantier et al., 2005; Smith et al., 2001).

### *3.3.3 Antibodies*

Antibodies against phosphotyrosine (PY20, 4G10); the AChR β subunit (mAb35); the conserved C-terminus of Src, Fyn, and Yes (*src*-CT); MuSK antibody; rapsyn; and β-dystroglycan were all used as previously described (Marangi et al., 2001; Mittaud et al., 2001; Moransard et al., 2003). Human anti-MuSK serum was obtained from Dr. Angela Vincent, Oxford, UK. It originates from myasthenia gravis patients who had high concentrations of anti-MuSK antibodies in their bloodstream. Experiments for Figure 3.4 were carried out with this serum and also with rabbit anti-MuSK antibodies (Fuhrer et al., 1997). Sequence-specific phosphorylation of SFKs (at $Y_{418}$) was detected by rabbit polyclonal phosphopeptide-specific antibody purified by sequential epitope-specific chromatography as performed by the supplier (BioSource Europe, S.A., Brussels, Belgium; Mittaud et al., 2004). SHP-2 antibody (sc-7384; Santa Cruz Biotechnology Inc., CA, USA); Alexa Fluor® 488 anti-mouse and Alexa Fluor® 546 anti-rabbit (Molecular Probes, Eugene, OR, USA); rabbit polyclonal c-Myc antibody (sc-789; Santa Cruz Biotechnology Inc., CA, USA); and mouse monoclonal Flag M2 antibody (F3165; Sigma, Switzerland) were used as indicated by the suppliers.

### *3.3.4 Assay for stability of AChR clusters*

To analyse the effect of pervanadate on spontaneous or on agrin-induced AChR clusters in C2 myotubes, cultures were incubated with pervanadate for 30 minutes, after which agrin (1 nM) was added for 6-8 hours. In *src*$^{-/-}$*;fyn*$^{-/-}$ and corresponding wild-type cells, myotubes were pre-incubated with pervanadate for 30 minutes, followed by addition of agrin (1 nM) for 16 h. In controls, either pervanadate, agrin, or both, were omitted during the same incubation times. To study the effect of pervanadate on the stability of agrin-induced AChR clusters, C2 myotubes (after 6 hours of agrin treatment) were subsequently washed twice with fusion medium and

maintained in fusion medium lacking agrin for an overnight period, in the presence or absence of pervanadate. This procedure was shown to be efficient in removing the vast majority of agrin from cells (Mittaud et al., 2004). In *src*$^{-/-}$;*fyn*$^{-/-}$ and corresponding wild-type cells, following an overnight treatment with agrin, cultures were washed twice with DM fusion medium and maintained in fusion medium for 5 hours, in the presence or absence of pervanadate.

To study the requirement for both SFKs and PTPs for the stability of agrin-induced AChR clusters, cultures were treated with agrin for 6-8 hours. Src-class kinase inhibitor PP2 (10 μM) was added to cultures during the last 2 hours of agrin treatment (Charpantier et al., 2005; Smith et al., 2001). Cultures were then washed twice with fusion medium followed by a 16 h incubation in fusion medium including PP2, or PP2 and pervanadate.

### *3.3.5 AChR clustering assay and quantification of clusters*

To study the effects of inhibitors, and of SHP-2 shRNA on AChR cluster formation or stability, AChR clusters were visualised by incubating myotube cultures grown in 3.5 cm dishes with 100 nM tetramethylrhodamine-conjugated α-bungarotoxin (α-BTX) (Molecular Probes) in fusion medium for 1 hour at 37°C followed by fixation in PFA in potassium phosphate buffer containing 11% sucrose for 15 minutes at room temperature, or in methanol for 7 minutes at -20°C (Marangi et al., 2001). Myotubes were examined at 400X magnification in both rhodamine and fluorescein channels with a fluorescence microscope (Axioskop II; Zeiss). Representative pictures (1344 x 1024 pixels) were taken and processed with a cooled digital camera (Orcacam; Hamamatsu) and SimplePCI software run on a Dell Dimension 8300 computer. The exposure times for individual channels were kept constant in all experiments.

AChR clusters were quantified using the NIH ImageJ 1.34 software. Images were opened in ImageJ, the scale was set to 3.8 pixels/μm for pictures taken at 400X, and to 1.9 pixels/μm for pictures taken at 200X. The threshold levels were set to 150-255, depending on the general background. Particles were analysed, with the minimum particle size defined at 100 pixels. Data extracted from each picture included

minimum and maximum particle size, mean particle size, mean particle area and number of particles. Particles >100 pixels approximated very closely to what would be considered as a cluster >10 μm length as judged by eye, and were thus taken as representative of a single AChR cluster. AChR clusters were quantified from approximately 15 random fields per experiment, and the mean ± SEM number of clusters per field was determined.

### *3.3.6 Immunoprecipitations and immunoblot analysis*

To examine the effects of inhibitor treatments in the presence or absence of agrin, and following agrin withdrawal, on the phosphorylation status of the AChR β subunit, MuSK, and on the activity-dependent phosphorylation of Src $Y_{418}$, immunoprecipitations were carried out from myotube cultures. To precipitate AChRs, cell lysates from cells grown on 6 or 10 cm plates were treated as previously described (Fuhrer et al., 1997; Mittaud et al., 2004), and AChRs precipitated using biotin-coupled α-BTX followed by streptavidin-coupled agarose beads (Molecular Probes, Leiden, The Netherlands). MuSK was precipitated as previously described (Fuhrer et al., 1997; Mittaud et al., 2004). Src was precipitated using monoclonal anti-v-Src antibody coupled to Protein G Sepharose beads (GE Healthcare, Uppsala, Sweden). Following immunoprecipitations, all samples were loaded on sodium dodecyl sulphate (SDS)-polyacrylamide gels and probed using a mixture of phosphotyrosine antibodies 4G10 and PY20 for AChR β and MuSK, or Src $pY_{418}$ antibody for SFKs. Quantitations of immunoblots were done by scanning exposed films containing grey, nonsaturated signals with a computerised densitometer (HP Scanjet 5530) and using the NIH ImageJ 1.34 software. Experiments were repeated three times, to obtain consistent results.

To analyse the effects of SHP-2 knockdown on the endogenous levels of SHP-2 and several other postsynaptic proteins, mature transfected myotubes were lysed and loaded onto a SDS gel. Following transfer to nitrocellulose paper, postsynaptic proteins were probed using specific antibodies against rapsyn, β-DG, MuSK, SHP-2, AChR β subunit and Src-CT.

### *3.3.7 Expression constructs and shRNA*

Myc-tagged wild-type MuSK expression construct, *MuSK-myc* (*pMuSK_myc*), was a gift of Dr. H.R. Brenner (Department of Physiology, University of Basel, Switzerland) (Jones et al., 1999). Wild-type SHP-2 (obtained from Dr. J.L. Bixby (University of Miami School of Medicine, Miami, USA)) (Chen et al., 2002) was subcloned into *Bam/Not* of *pcDNAI-Flag* (a gift of Dr. M. Gesemann, Brain Research Institute, University of Zurich, Switzerland) to obtain a *SHP-2-Flag* (*pcDNAI-SHP-2-Flag*) construct. The constructs were transfected into COS cells using Fugene6 (Roche, Basel, Switzerland) as described below.

*pSUPER.neo+gfp* vector (*pSUPER*) was purchased from OligoEngine (Seattle, USA). shRNA (short-hairpin RNA) constructs against murine SHP-2 (NCBI Accession number NM_011202) were generated by cloning the following target sequences into the *BglII/XhoI* sites of the *pSUPER* vector: (1) 5'-gaatacggggtcatgcgtgtt-3', (2) 5'-gaacactggggactactatga-3' and (3) 5'-aaatgtgtcaagtactggcct-3'. Target sequence (1) was adapted from Higuchi et al. (2004) and target sequences (2) and (3) were adapted from Kontaridis et al. (2004).

### *3.3.8 Transfections*

Transfections of constructs into C2 and COS cells were carried out using Fugene6 transfection reagent (Roche, Basel, Switzerland) according to the manufacturer's recommendations. Optimal transfection conditions were established by transfecting C2 myoblasts at 70-90% confluence. Two days later cells were induced to fuse by switching to fusion medium for 2 days. Transfected cells were observed by the expression of green fluorescence protein (GFP). Transfection efficiency on the myoblast stage was at 40-50%, and at the myotube stage (following fusion) was estimated at 80-100%. For co-transfection of COS cells with *MuSK-myc* and *SHP-2-Flag* constructs, equal quantities of DNA were transfected.

### *3.3.9 $^{125}$I-α-bungarotoxin binding assay*

To measure the surface level of AChRs following SHP-2 knockdown by shRNA, myotube cultures grown in 3.5 cm plates were incubated with $^{125}$I-α-BTX (Amersham Biosciences, Arlington Heights, IL) for 1 hour at 37°C. Cultures were washed twice with cold PBS supplemented with 1 mM Na orthovanadate and 50 mM NaF and lysed at 4°C in 1 ml lysis buffer (Fuhrer et al., 1997) for 15 minutes. Cell lysate aliquots were taken for protein determination assays. Radioactivity from surface AChR-bound $^{125}$I-α-BTX was counted in an LKB Wallac 1282 CompuGamma counter (2 min counting mode). Non-specific binding was determined using 1 μM unlabelled α-BTX (1 hour pre-incubation and during radioactive labelling) (Charpantier et al., 2005). Radioactive signals were robust (30000-100000 cpm), and the non-specific background was low (5-10%). Counts were normalised for the total protein content for each sample. AChR surface levels following SHP-2 knockdown by shRNA were plotted as percentages of *pSUPER* transfected controls.

## 3.4 Results

### *3.4.1 PTP inhibition by pervanadate reduces the number of agrin-induced AChR clusters, and the stability of these clusters following agrin removal in wild-type myotubes*

To address the role of PTPs in spontaneous and agrin-induced AChR clustering, and the stability of clustered AChRs, we sought to use the potent PTP inhibitor sodium pervanadate (Pumiglia et al., 1992; Wallace, 1995). To study its effect on spontaneous AChR clustering 20 μM of freshly-prepared pervanadate was applied to C2 myotubes for a period of 6-8 hours, following which the AChR clusters were stained with rhodamine-α-bungarotoxin (BTX) and visualised by fluorescence microscopy. We took representative fields from each treatment, and the number of AChR clusters was quantified automatically using ImageJ. We set the image threshold levels to 150-255, and the minimum pixel area of an AChR cluster at 100 pixels. PTP inhibition did not lead to any change in the number of AChR clusters observed per field when compared to the untreated control (Figure 3.1). It led however to the appearance of many low intensity areas of AChRs (Figure 3.1A). Since these were clearly fainter than typical clusters and hard to quantify using threshold levels (due to poor signal-to-noise ratio), we did not consider these structures for our cluster quantifications. To analyse the effect on agrin-induced AChR clustering, we applied pervanadate to wild-type myotubes for 30 min, followed by a 6-8 h addition of 1 nM agrin. This resulted in a significant decrease in the number of agrin-induced AChR clusters (Figure 3.1). Again, low density AChR areas were neglected. The stability of AChR clusters can be modelled in cell culture by the withdrawal and washing off of agrin for a number of hours following a full agrin induction (Sadasivam et al., 2005; Smith et al., 2001). Following agrin withdrawal for 16 h we saw a significant loss in the number of AChR clusters. This stability of clusters was significantly reduced when PTPs were blocked by pervanadate at the point of agrin removal (Figure 3.1A and B). These results show that phosphatase activity of PTPs is required both during the agrin induction of AChR clusters, as well as for the stability of these clusters following removal of agrin.

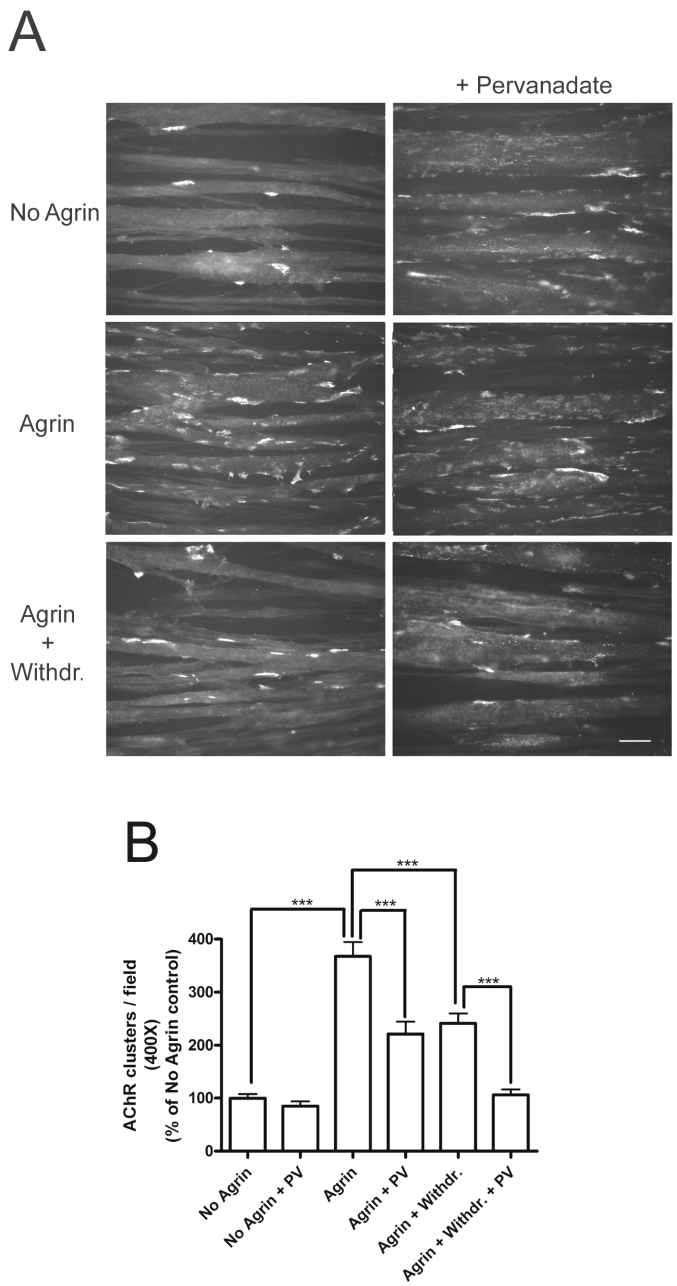



**Figure 3.1. PTP inhibition by pervanadate reduces the number of agrin-induced AChR clusters, and their stability following agrin removal in wild-type myotubes.** (A) PTPs were blocked in C2 myotube cultures for 30 min with 20 μM pervanadate prior to addition of 1 nM agrin for 6-8 h (Agrin + PV); or at the point of agrin withdrawal (Agrin + Withdr. + PV), for a withdrawal period of 16 h. In controls, cultures were left untreated (No Agrin), treated with pervanadate alone for 6-8 h (No Agrin + PV), agrin alone for 6-8 h (Agrin), or treated with agrin followed by withdrawal for 16 h (Agrin + Withdr.). AChRs were stained with rhodamine-α-BTX and analysed by fluorescence microscopy. Scale bar, 40 μm. (B) The number of AChR clusters per field was calculated using ImageJ software, using fixed intensity thresholds (150-255) and minimum area of 100 pixels occupied by a cluster. The number of AChR clusters per field (400X magnification) is shown as the percentage of untreated cells (No Agrin) (means ± SEM, $N = 50$ from four similar experiments). Phosphatase inhibition decreases significantly both the number of agrin-induced AChR clusters, and the stability of these clusters following agrin removal (*** $p < 0.0001$; two-tailed paired $t$ test).

### *3.4.2 The instability of AChR clusters in the absence of SFKs is rescued by pervanadate inhibition of PTPs*

The importance of two families of PTKs during agrin-induced clustering of AChRs, and in the stability of agrin-induced clusters, has been recently shown. Abl kinases are required during agrin-induced clustering of AChRs (Finn et al., 2003), and for enhancing MuSK phosphorylation (Finn et al., 2003; Mittaud et al., 2004). SFKs phosphorylate both MuSK and the AChR β subunit (Mittaud et al., 2004; Mohamed et al., 2001), and are important for stability of AChR clusters at the NMJ *in vivo* and *in vitro* (Sadasivam et al., 2005; Smith et al., 2001). It is therefore of great interest to explore the interplay between phosphatase and SFK activities in the stability of AChR clusters. By using *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes we investigated the effect of phosphatase inhibition by pervanadate on this process. The absence of Src and Fyn allows for normal clustering of AChRs by agrin. However these clusters have a shortened half-life (Smith et al., 2001), and are less stable upon agrin withdrawal (Figure 3.2A, B). We therefore investigated the role of phosphatase activity in the absence of Src and Fyn (in *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes). We inhibited phosphatase activity by pervanadate in the absence of agrin, during agrin induction, and following removal of agrin. We then analysed the effects on AChR clusters (Figure 3.2A, B). We observed a slight, but not significant increase in the level of spontaneous and agrin-induced AChR clustering. Most dramatically, the stability of agrin-induced AChR clusters upon withdrawal of agrin was completely rescued when pervanadate was added at the time of agrin removal. These results suggest that in the absence of Src and Fyn, PTPs do not modulate spontaneous or agrin-induced clustering of AChRs. However, phosphatase activity has a destabilising effect on pre-existing agrin-induced clusters in *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes, since inhibiting this activity by pervanadate rescues AChR stability after removal of agrin.

Src and Fyn are not the only members of this family of kinases present in myotubes. A third member of the SFKs, Yes, has been shown to be upregulated in *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes (Smith et al., 2001). This led us to investigate whether the observed destabilising effect of phosphatases was solely due to the absence of Src and Fyn, or

whether this effect would also occur when all members of the SFKs were blocked. We therefore made use of the highly-specific inhibitor PP2 to block SFKs in C2 wild-type myotubes (Hanke et al., 1996). We again stimulated AChR clustering by agrin, and removed agrin to analyse the effect on the stability of these AChR clusters. We found that PP2 had no effect on the level of clustering induced by agrin (Figure 3.2C, D), as has been previously observed (Smith et al., 2001). However, on removal of agrin, blocking SFKs with PP2 led to a two-fold decrease in the number of remaining AChR clusters. In previous studies, the inhibition of SFKs using CGP77675 did not affect the stability of pre-existing agrin-induced clusters (Mittaud et al., 2004). This different outcome may be caused by the different specificity of CGP77675 and PP2 in inhibiting individual SFK members. Another reason is that Mittaud et al. (2004) added CGP77675 at the very time of agrin removal and not 2 h before, as we did. Our observed destabilisation of clusters by PP2 paralleled the effect of the absence of Src and Fyn on cluster stability, as seen in *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes (Figure 3.2 A). We then proceeded with blocking PTPs upon agrin withdrawal in PP2-treated myotubes to see in what way phosphatase inhibition would influence the AChR clusters when SFKs are inactivated. Indeed we observed a complete rescue of AChR cluster stability by inhibiting phosphatase activity with pervanadate (Figure 3.2C, D). From these results we can conclude that the activity of PTPs could indeed be the key destabilising factor for agrin-induced AChR clusters in the absence of SFK activity. Thus, the stability of agrin-induced AChR clusters following the withdrawal of agrin appears to require a fine balance between the kinase activities of SFKs, and the phosphatase activities of PTPs.

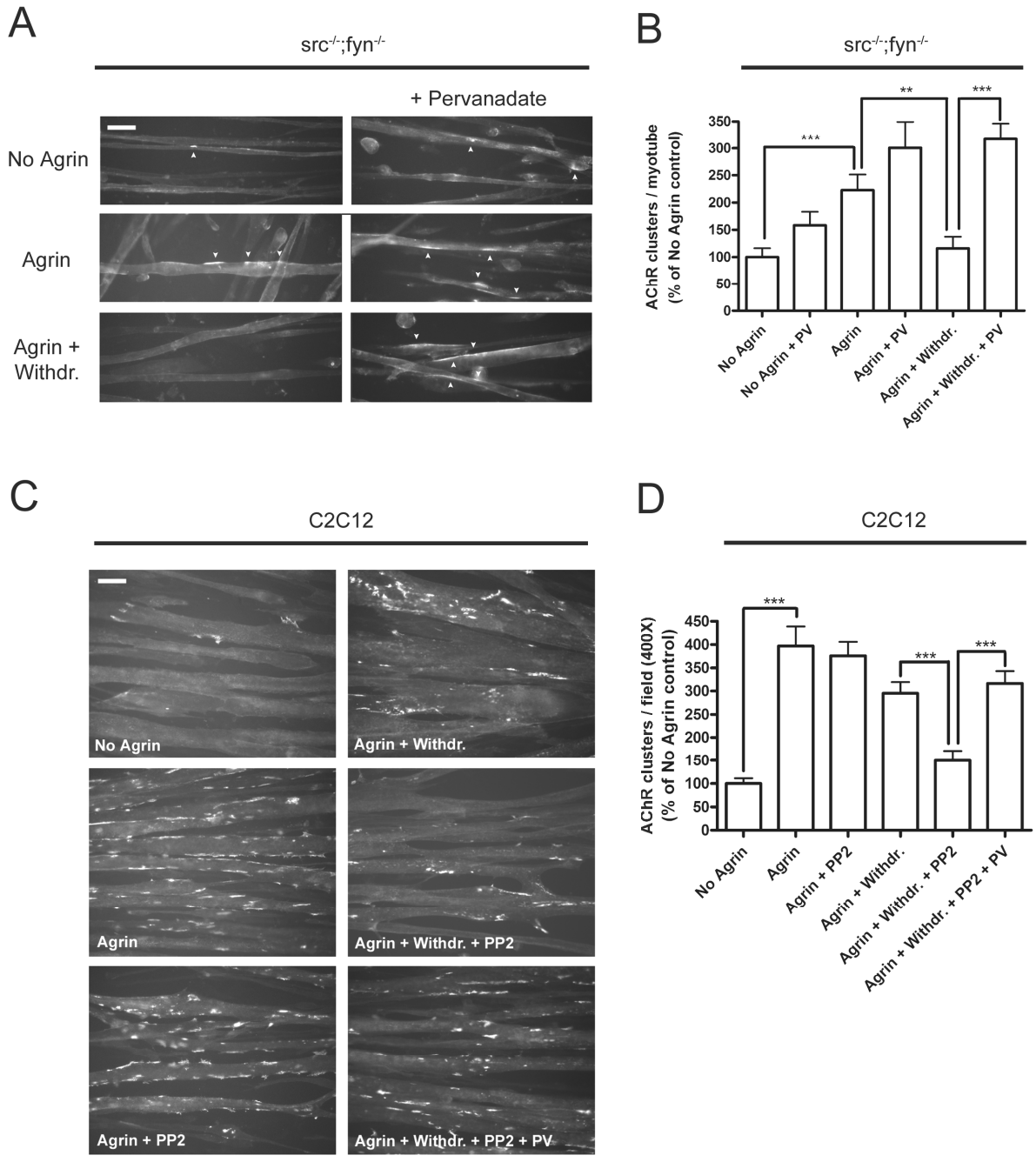

A
src-/-;fyn-/-
+ Pervanadate
No Agrin
Agrin
Agrin + Withdr.
B
src-/-;fyn-/-
AChR clusters / myotube (% of No Agrin control)
No Agrin
No Agrin + PV
Agrin
Agrin + PV
Agrin + Withdr.
Agrin + Withdr. + PV
C
C2C12
No Agrin
Agrin + Withdr.
Agrin
Agrin + Withdr. + PP2
Agrin + PP2
Agrin + Withdr. + PP2 + PV
D
C2C12
AChR clusters / field (400X) (% of No Agrin control)
No Agrin
Agrin
Agrin + PP2
Agrin + Withdr.
Agrin + Withdr. + PP2
Agrin + Withdr. + PP2 + PV

**Figure 3.2. Rescue of the instability of AChR clusters in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes and PP2-treated C2 myotubes by pervanadate inhibition of PTPs.** (A) PTPs were blocked with 20 μM pervanadate in *src*$^{-/-}$;*fyn*$^{-/-}$ myotube cultures for 30 min prior to addition of 1 nM agrin for 16 h (Agrin + PV), or at the point of agrin withdrawal for a period of 5 h (Agrin + Withdr. + PV). Controls included untreated myotubes (No Agrin), agrin treatment for 16 h (Agrin) and agrin treatment followed by withdrawal for 5 h (Agrin + Withdr.). AChRs were stained with rhodamine-α-BTX and analysed by fluorescence microscopy. Scale bar, 40 μm. White arrowheads indicate AChR clusters. (B) The number of AChR clusters per myotube is shown as the percentage of untreated cells (No Agrin) (means ± SEM, $N = 15$ from 2 similar experiments). (C) Src-family kinase inhibitor PP2 was used to block SFKs in C2 myotubes. Myotubes were treated with 10 μM PP2 for 90 min prior to agrin addition for 6-8 h (Agrin + PP2). To analyse the effect on stability of AChR clusters, PP2 treatment was started 2 h prior to agrin withdrawal, after which pervanadate (20 μM) was added to the medium at the time of agrin removal, for 16 h (Agrin + Withdr. + PP2 + PV). In controls, cultures were left untreated (No Agrin), treated with agrin +/- PP2 pre-treatment for 6-8 h (Agrin; Agrin + PP2), or treated with agrin followed by withdrawal for 16 h +/- PP2 pre-treatment (2 h before agrin removal) (Agrin + Withdr.; Agrin + Withdr. + PP2). AChRs were stained with rhodamine-α-BTX, analysed by fluorescence microscopy. Scale bar, 40 μm. (D) The number of AChR clusters per field (400X magnification) was calculated, as described in Figure 3.1, as the percentage of untreated cells (No Agrin) (means ± SEM, $N = 30$ from three experiments; ** $p < 0.01$, *** $p < 0.001$; two-tailed paired *t* test).

### *3.4.3 The inhibition of phosphatases by pervanadate increases AChR β subunit phosphorylation but has no effect on MuSK phosphorylation*

Since the role of phosphatases is to dephosphorylate tyrosine residues on substrates and also on PTKs, we sought to investigate closer the mechanism by which PTP activity would be affecting the clustering and stability of AChRs following MuSK stimulation by agrin. For this purpose, in C2 myotube cultures, we stimulated for 6-8 h with agrin, and subsequently removed agrin for 16 h, in the presence or absence of pervanadate. Following this, we proceeded with precipitating AChRs and MuSK from cell lysates, and probed for the phosphorylation status of these isolated postsynaptic proteins using phosphotyrosine-specific antibodies. We found that the phosphorylation levels of AChR β subunit and MuSK are strongly increased by agrin stimulation (Figure 3.3A, B). The phosphorylation of AChR β subunit remains elevated 16 h following agrin removal, while MuSK phosphorylation disappears. Surprisingly, we found that by inhibiting phosphatases with pervanadate, an increase in AChR β subunit phosphorylation was observed both during agrin stimulation, and following agrin withdrawal (Figure 3.3A). Agrin-induced MuSK phosphorylation was however left unchanged following pervanadate treatment (Figure 3.3B). These results

show that in our C2 myotubes phosphatases control the phosphorylation status of the AChR β subunit, but not of MuSK, both during agrin stimulation and following its withdrawal. However this increase in AChR β subunit phosphorylation has no positive effect on the stability of AChR clusters, since pervanadate renders clusters less stable in C2 myotubes (Figure 3.1). Increasing the phosphorylation of AChR β is this not sufficient to stabilise clusters.

In *src*$^{-/-}$;*fyn*$^{-/-}$ and their corresponding wild-type myotube cultures, we also investigated the effects of pervanadate-mediated phosphatase inhibition on the phosphorylation status of the AChR β subunit during or following agrin treatments. We find that phosphorylation levels of the AChR β subunit are boosted by agrin, and stay up after agrin withdrawal for 5 h (Figure 3.3C). In *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes treated with agrin overnight, AChR β phosphorylation decreases within 5 h of agrin withdrawal (Sadasivam et al., 2005). The difference to our present experiments (Figure 3.3C) appears to be the time of agrin incubation (8 h) which results in stable phosphorylation (Figure 3.3C). Consistent with this, a 5 min pulse of agrin results in efficient AChR β subunit phosphorylation even when agrin is withdrawn for 8 h. One parameter for stability of AChR β subunit phosphorylation in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes may thus be the duration of agrin treatment. After 8 h, the phosphorylation is stable but after overnight treatment it is not (Sadasivam et al., 2005). More importantly, we found that phosphatase inhibition of *src*$^{-/-}$;*fyn*$^{-/-}$ and their corresponding wild-type myotube cultures leads to a similar increase in phosphorylation of the AChR β subunit both following agrin stimulation, and after agrin withdrawal. The increase is however smaller than in C2 myotubes. From these results we can conclude that the PTP control of the phosphorylation status of the AChR β subunit does not correlate with the effects of PTPs on the stability of agrin-induced AChR clusters following agrin withdrawal. Thus, phosphorylations on other targets may play a role in cluster stabilisation.

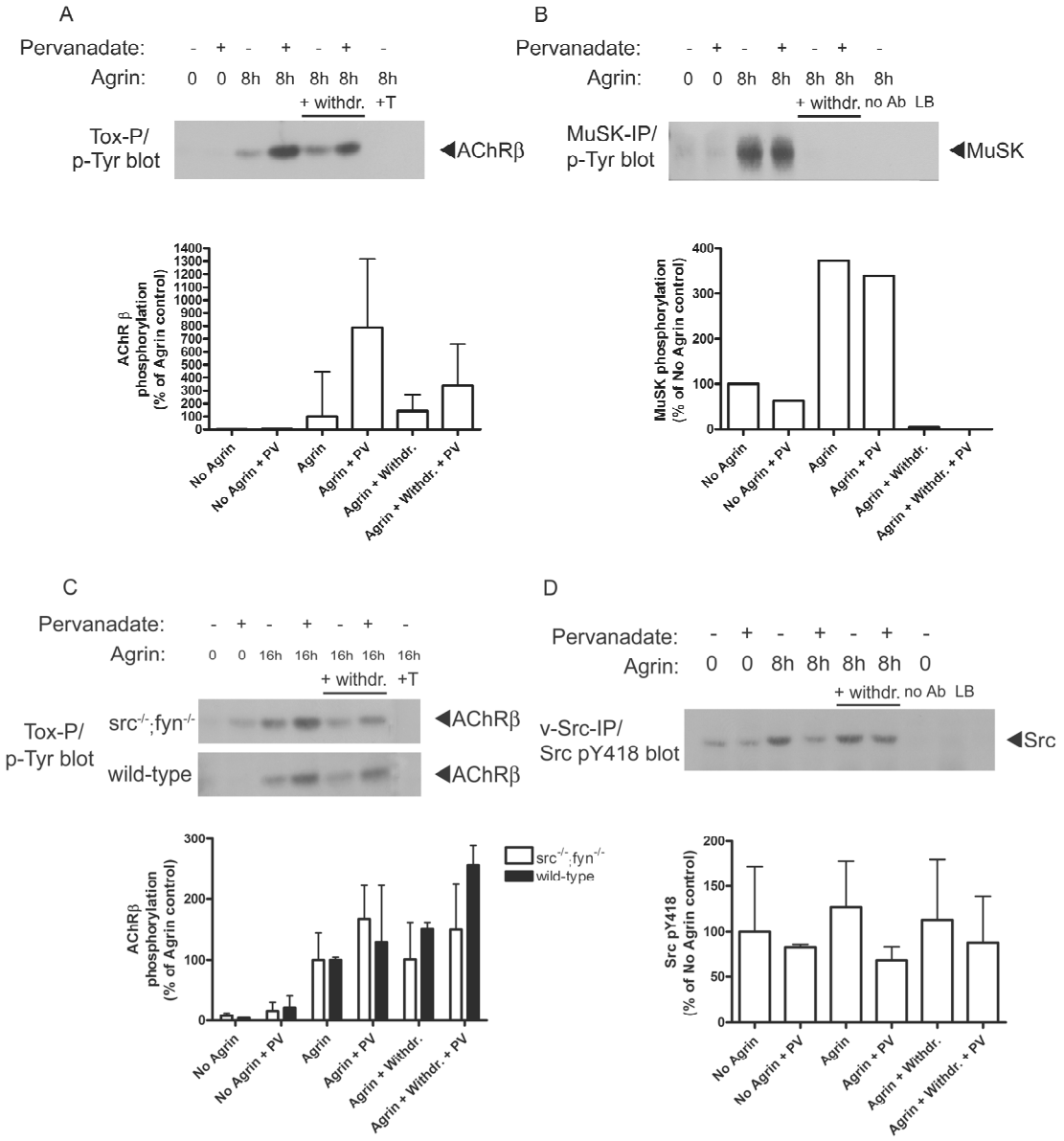



**Figure 3.3. The inhibition of phosphatases by pervanadate increases AChR β subunit phosphorylation, decreases Src activity, but has no effect on MuSK phosphorylation.** C2, *src*$^{-/-}$*;fyn*$^{-/-}$ or corresponding wild-type myotubes were treated with agrin in the presence or absence of pervanadate, as described in Figure 3.1A and Figure 3.2A respectively. Agrin withdrawal was for 16 h in A, B, and D, and for 5 h in C. Immunoprecipitations of the AChR (A, C), MuSK (B) and Src (C) were carried out followed by anti-phosphotyrosine (A-C) or anti-Src pY418 (D) immunoblotting. (A) In C2 myotubes, phosphatase inhibition by pervanadate increases the phosphorylation level of AChR β subunit both following agrin treatment and following agrin withdrawal compared to untreated controls. (B) In C2 myotubes, MuSK phosphorylation is increased strongly upon agrin stimulation, and falls to background levels after agrin withdrawal. In our cultures, phosphatases do not appear important in controlling agrin-induced MuSK phosphorylation since pervanadate had no effect in altering MuSK phosphorylation levels. (C) Pervanadate also slightly increases the phosphorylation level of AChR β subunit in both *src*$^{-/-}$*;fyn*$^{-/-}$ or corresponding wild-type myotubes following agrin treatment and following agrin withdrawal as compared to untreated controls. (D) In C2 myotubes, Src activity is slightly increased upon stimulation of myotubes by agrin, as judged by the phosphorylation state of activity-specific $Y_{418}$. Src activity is decreased upon pervanadate inhibition, implying one or more phosphatases as positive regulators of Src activity mostly during agrin stimulation but also after its withdrawal.

### *3.4.3 PTPs control Src activity*

The activity of SFKs is controlled by the phosphorylation status of a number of key tyrosine residues. The main autophosphorylation site of SFKs is in their activation loop. The phosphorylation of this $Y_{418}$ is an important feature of SFK activation (Thomas and Brugge, 1997). Dephosphorylation of a C-terminal $Y_{529}$ leads to maximal and sustained activity of SFKs, by displacing the intramolecular interaction between $pY_{529}$ and the SH2 domain. This enhances kinase reconfiguration and activity (Thomas and Brugge, 1997). Probing for the phosphorylation status of $Y_{418}$ therefore allows for an assessment of SFK activation. We proceeded to investigate whether PTP inhibition by pervanadate would affect the activity of SFKs during agrin stimulation or following agrin withdrawal from C2 myotube cultures. We treated C2 myotube cultures as already described above (Figure 3.3), followed by precipitation of Src from cell lysates. We then probed with Src $pY_{418}$-specific antibodies for Src activity in the presence or absence of phosphatase inhibition. We found that Src activity (reflecting the total cellular Src pool) is slightly enhanced following agrin stimulation (Figure 3.3D). We then analysed the effects of inhibiting phosphatases with pervanadate on Src $pY_{418}$ during agrin induction and withdrawal. We find that blocking PTP activity leads to a slight reduction in the phosphorylation of Src $Y_{418}$. This block is most pronounced in the agrin induction phase and weaker in the withdrawal phase. Thus, the activity of Src during agrin induction and following agrin withdrawal may be positively controlled by one or more PTPs, which upon inhibition by pervanadate lead to decreased activity of Src. Src activity is required for AChR cluster stability. Therefore, upon inhibition of SFKs by PP2, AChR clusters are destabilised (Figure 3.2; Sadasivam et al., 2005). Since PTP activity may also control SFK activity, as suggested in Figure 3.3D, inhibiting PTPs tends to result in slightly reduced SFK activity. In wild-type C2 cultures, this may be a reason for unstable clusters. However, in PP2-treated wild-type cultures, or *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes, the effect of PTP inhibition is the reverse, that is to increase the stability of the already unstable clusters. It remains to be investigated whether pervanadate decreases or increases the activity of the remaining SFKs (Yes) in *src*$^{-/-}$;*fyn*$^{-/-}$ cells. In any case, for

proper stability of AChR clusters to be maintained following agrin induction, a balance between the activities of SFKs and PTPs is crucial.

### *3.4.4 Protein tyrosine phosphatase SHP-2 increasingly associates with MuSK upon agrin stimulation*

An example of how the activity of a PTP is required in positively controlling the actions of a kinase is the regulation of Src activity by the SH2 domain-containing protein tyrosine phosphatase SHP-2 (Zhang et al., 2004). SHP-2 is enriched at neuromuscular synapses, and colocalises with AChRs *in vivo* (Tanowitz et al., 1999). SHP-2 is therefore a likely candidate PTP playing roles in controlling PTK and/or SFK activity during agrin-induced signalling leading to AChR clustering, and during the stabilisation of AChR clusters thereafter. In cultured myotubes, 0.5 nM agrin stimulates MuSK phosphorylation to its maximal levels after only 40 min of incubation. For this reason we sought to investigate whether SHP-2 could be associating with MuSK upon MuSK activation. We treated C2 myotubes with agrin for 40 minutes, following which we immunoprecipitated MuSK and probed for both MuSK and SHP-2. In the control (Ctl), the MuSK antibody was omitted (Figure 3.4A). We find that SHP-2 is co-immunoprecipitated together with MuSK, and that the association between the two increases 2.3-fold following MuSK phosphorylation by agrin.

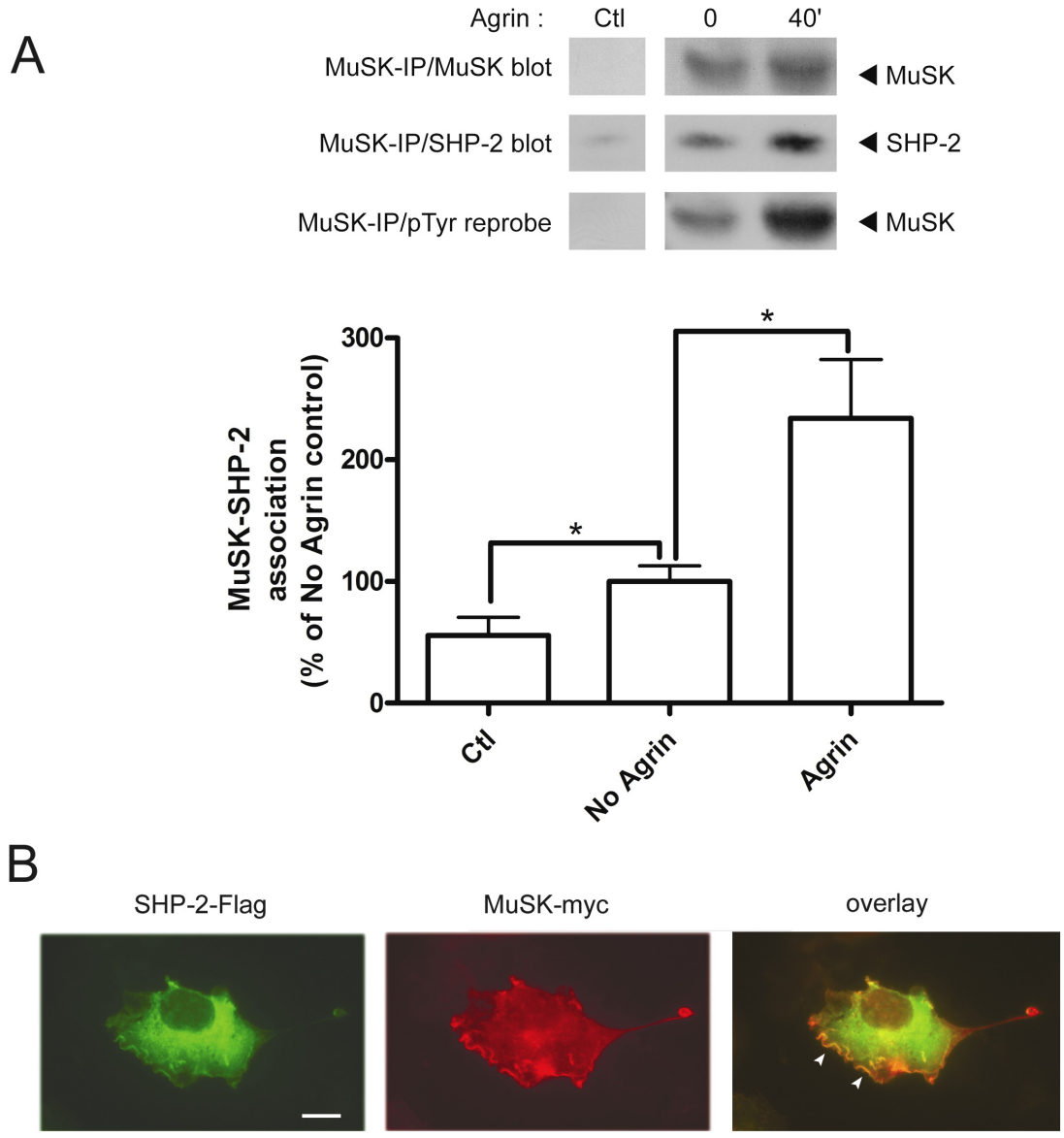



**Figure 3.4. Protein tyrosine phosphatase SHP-2 increasingly associates with MuSK upon agrin stimulation.** (A) C2 myotubes were treated with 0.5 nM agrin for 40 min. Following MuSK immunoprecipitation, immunoblotting for SHP-2 and MuSK was carried out, followed by reprobing for phosphorylation of MuSK. As control, the MuSK antibody was omitted (Ctl). Levels of SHP-2 co-precipitated with MuSK are shown (normalised for the amount of precipitated MuSK) as percentage of no agrin control. Co-immunoprecipitation of SHP-2 with MuSK indicates its association with MuSK, which increases significantly upon agrin-induced MuSK phosphorylation (means ± SEM, from seven experiments; * $p < 0.05$; two-tailed unpaired *t* test). (B) COS cells were co-transfected with wild-type SHP-2-Flag and MuSK-myc constructs. Cells were then fixed and stained with anti-Flag/Alexa488 and anti-myc/Alexa546 antibodies. SHP-2 localises throughout the whole of the cytoplasm, while MuSK localisation is concentrated along the plasma membrane. White arrowheads in the overlay indicate a colocalisation of MuSK and SHP-2 along membrane ruffles on the plasma membrane. Scale bar, 40 μm.

We proceeded to investigate this association between MuSK and SHP-2 further using a heterologous cell system. We transfected COS cells with *myc*-tagged wild-type MuSK and *Flag*-tagged wild-type SHP-2 constructs. We then visualised MuSK and SHP-2 in the transfected cells by immunocytochemistry using anti-myc/Alexa546 and anti-Flag/Alexa488 antibodies. We found that, as expected, being a ubiquitously-expressed non-receptor PTP, SHP-2 localises throughout the whole of the cell, but also along membrane ruffles on the plasma membrane (Figure 3.4B). On the other hand, MuSK shows a membrane localisation, characteristic for a RTK. Both SHP-2 and MuSK colocalised within these membrane ruffles present along the plasma membrane. They however did not cause the delocalisation of one another, since singly-transfected COS cells also exhibited similar distribution patterns of the expressed proteins (data not shown). From these results we can conclude that MuSK and SHP-2 localise in similar regions along the plasma membrane. They also associate with one another, and SHP-2 increases its association to MuSK upon MuSK activation by agrin. This raises the possibility that SHP-2 associates to MuSK via one or both of its Src homology 2 (SH2) domains, since SH2 domains associate with phosphorylated tyrosine residues on membrane-bound RTKs, and in SHP-2, the engagement of the N-SH2 domain is required for its activation (Feng, 1999; Hof et al., 1998). If the association with MuSK is direct, this could lead to the activation of SHP-2 allowing it to act directly upon MuSK or to interact with downstream targets.

### *3.4.5 Knockdown of SHP-2 in myotubes by vector-driven shRNA specifically reduces SHP-2 protein levels, and has no effect on most other postsynaptic proteins*

In order to address the role of SHP-2 during clustering of AChRs in myotubes following agrin signalling, and in the stability of these clusters, we used a knockdown approach by RNA interference (RNAi). We cloned three short-hairpin RNA (shRNA) loops into the *pSUPER.gfp* vector, and tested their efficacy in downregulating SHP-2 expression in C2 myotubes. We used shRNA loops generated against three different

regions of the murine SHP-2 open-reading frame (ORF), based on three already published and successfully used siRNA or shRNA sequences (Higuchi et al., 2004; Kontaridis et al., 2004), and called these *pSUPER.shSHP2.1*, *pSUPER.shSHP2.2* and *pSUPER.shSHP2.3*. These shRNA constructs co-expressed EGFP, allowing easy identification of the successfully transfected myoblasts, and observation of the differentiation of these myoblasts into mature myotubes. Transfection efficiencies at the myoblast level were in the region of 40-50%, which upon differentiation and fusion led to close to 100% of the myotubes expressing EGFP (see Figure 3.6). By Western blot analysis of C2 myotube lysates we probed for the effective suppression of endogenous SHP-2 protein. Both *pSUPER.shSHP2.1* and *pSUPER.shSHP2.2* led to a very strong (> 60%) reduction of endogenous SHP-2 protein levels when compared to control transfected myotubes (*pSUPER* vector only) (Figure 3.5A). *pSUPER.shSHP2.3* appeared to have no effect. We therefore proceeded to analyse the effect of SHP-2 downregulation on other proteins present in muscles and important during AChR clustering and stability. We tested *pSUPER.shSHP2.*2 and found that SHP-2 knockdown using this construct had little or no effect on the expression of MuSK, Src, rapsyn, and β-dystroglycan (Figure 3.5B). However SHP-2 knockdown had a small yet significant effect in decreasing the expression of the AChR β subunit. To test whether this reduced expression of AChR β subunit would lead to pronounced change in the number of functional AChRs present at the surface of myotubes, we used radioligand binding of $^{125}$I-α-BTX to probe the functional surface AChRs following transfection with *pSUPER.shSHP2.2*. As seen in Figure 3.5C, we see a small but insignificant change in the number of surface functional AChRs (α-BTX binds with far higher affinity to intact and functional AChRs than to individual subunits; Tzartos and Changeux, 1983). This indicates that if SHP-2 has any effect on the AChR β subunit at the expression level, this reduced expression has little or no effect on the final level of functional receptors which are trafficked to the surface.

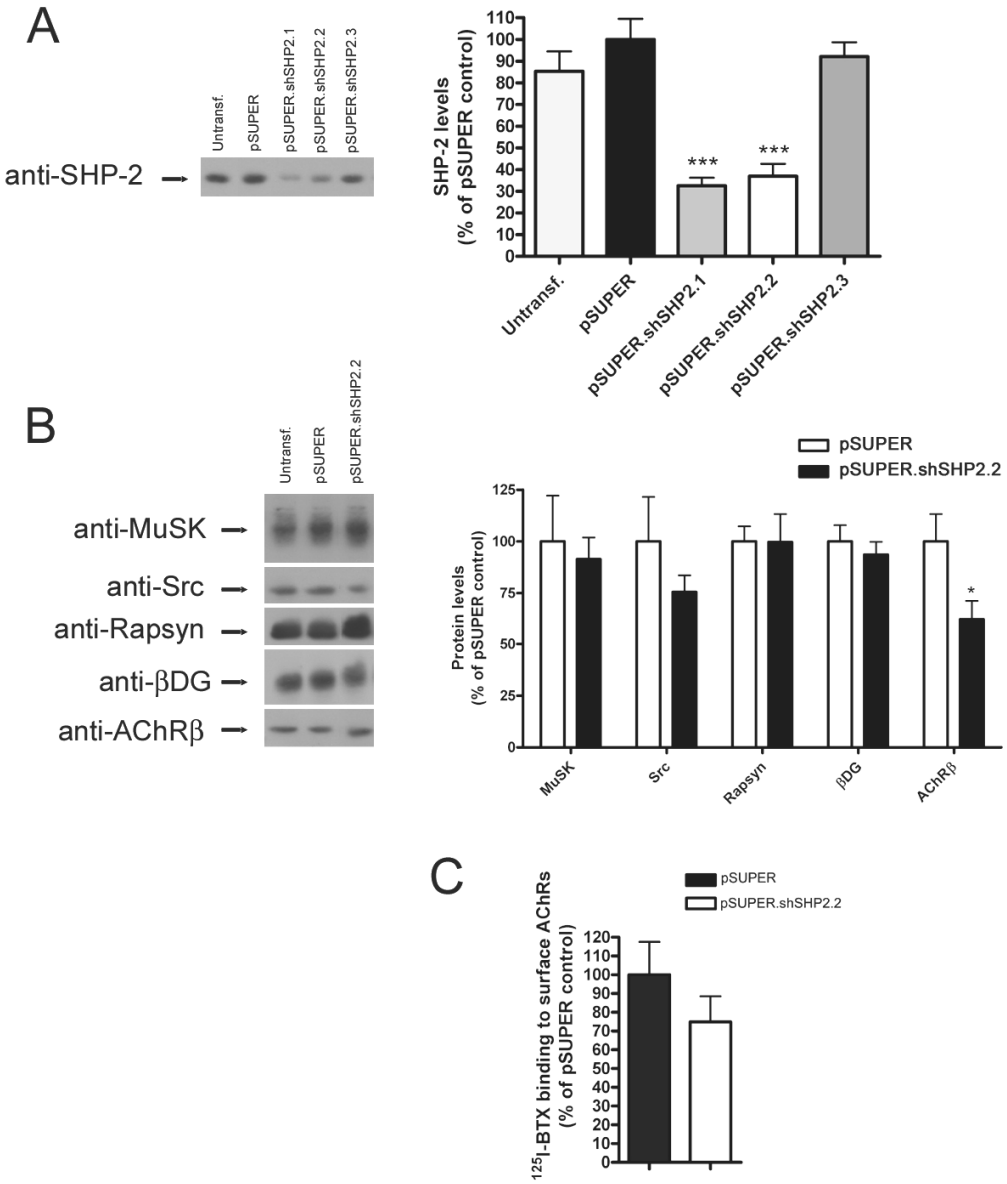



**Figure 3.5. Gene-specific targeting of SHP-2 in myotubes through vector-driven shRNA specifically knocks down SHP-2 levels, and has no effect on most postsynaptic proteins.** (A) C2 myotubes were transfected with either of three different *pSUPER*.shSHP2 constructs targeting specific regions of the murine SHP-2 ORF (NM_011202). No transfection (Untransf.) or *pSUPER* vector only (*pSUPER*) transfected myotubes served as controls. Western blot analysis of SHP-2 levels revealed that *pSUPER.shSHP2.1* and *pSUPER.shSHP2.2* knocked down > 60% of endogenous SHP-2, while *pSUPER.shSHP2.3* had no significant effect (only 8%). Remaining SHP-2 levels were calculated and are shown (as % of *pSUPER* vector only transfected myotubes) (means ± SEM, from eight experiments). (***$p < 0.0001$; two-tailed unpaired *t* test). (B) The effect of SHP-2 gene targeting on levels of several postsynaptic proteins was analysed by *pSUPER.shSHP2.2* transfection followed by Western blot analysis. This revealed that SHP-2 knockdown has no significant effect on levels of MuSK, Src, rapsyn, and β-dystroglycan. A significant effect was seen on AChR β subunit levels, which were down by approximately 40% of control transfected myotubes. Remaining protein levels were calculated and shown (as % of respective *pSUPER* vector only transfected myotubes) (means ± SEM, from eight experiments). (*$p < 0.05$; two-tailed unpaired *t* test). (C) Radioligand binding assays, using $^{125}$I-α-BTX binding to surface AChRs in intact C2 myotubes following SHP-2 knockdown, show that there is no significant effect of the absence of SHP-2 on the surface levels of AChRs, when compared to radioligand binding on control transfected myotubes.

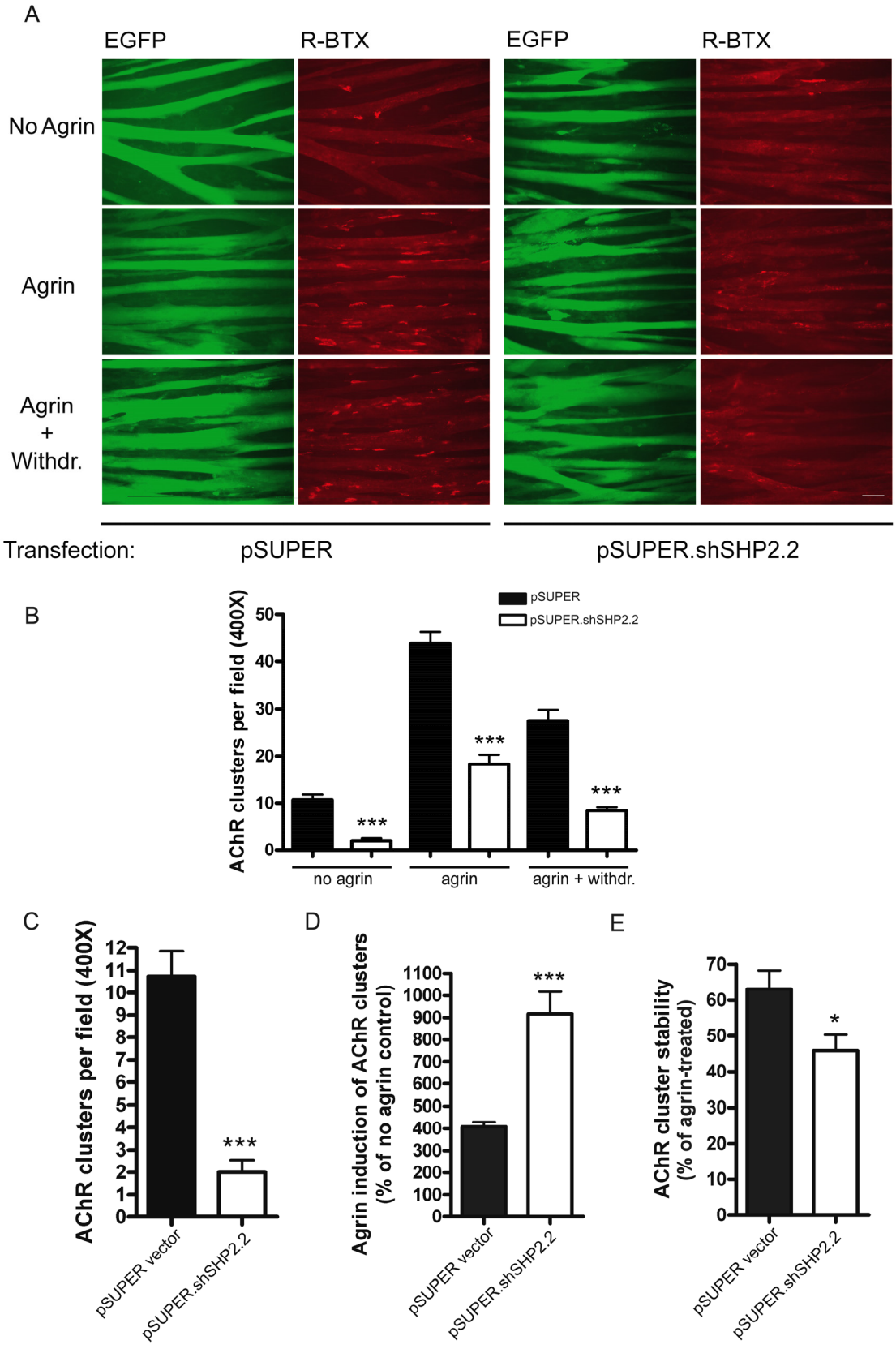
Analysis of *pSUPER.shSHP2.1* showed that apart from SHP-2, a slight downregulation of other postsynaptic proteins (rapsyn and β-dystroglycan) took place. Since SHP-2 knockdown is known to be involved in myogenesis (Kontaridis et al., 2004; Kontaridis et al., 2002), overall myotube differentiation may have been affected by this targeting vector. Therefore we concentrated our efforts on *pSUPER.shSHP2.2*.

### *3.4.6 SHP-2 is required for efficient AChR clustering, and for stability of clusters following agrin withdrawal*

To study the requirements of SHP-2 for AChR cluster formation and stability, we used C2 myotubes in culture as a model system. We suppressed SHP-2 by shRNA, as described above. To analyse effects on spontaneous AChR cluster formation, we then visualised AChR clusters using rhodamine-α-BTX and immunofluorescence microscopy. To analyse effects on agrin-induced clustering we treated cultures with agrin for 16 h, and proceeded to visualise the induced AChR clusters. To study the effects of SHP-2 knockdown on the stability of agrin-induced clusters, we withdrew agrin for 8 h following agrin stimulation, and finally proceeded to visualise the AChR clusters. As seen by the EGFP expression of myotubes in Figure 3.6A, transfection efficiencies at the myotube level were very high. Knockdown of SHP-2 expression resulted in a very strong effect at all levels of AChR clustering. Spontaneous AChR clusters were significantly reduced (Figure 3.6A, B, C), as were the number of agrin-induced AChR clusters (Figure 3.6A, B). Finally, even the stability of any agrin-induced AChR clusters was significantly affected in the absence of SHP-2, as seen following agrin withdrawal (Figure 3.6A, B). The effect of SHP-2 shRNA that we observed following induction by agrin was a reduction in the number of AChR clusters formed. However, the actual effect on agrin's induction potential was only obvious upon normalising the number of clusters formed following agrin stimulation to the number of AChR clusters present spontaneously before agrin induction. As seen in Figure 3.6D, we show that the actual effect of SHP-2 knockdown is to increase agrin's induction potential of AChR clusters 2.2-fold over that in control transfected

myotubes. To further analyse the actual effect of SHP-2 knockdown on the stability of agrin-induced AChR clusters, we normalised the number of AChR clusters following agrin withdrawal to the number of clusters that were induced by agrin. The result clearly shows that there is a significant decrease in the stability of AChR clusters following agrin withdrawal when SHP-2 expression is knocked down by shRNA (Figure 3.6E). From these results we therefore conclude that SHP-2 is required for muscles to be able to cluster AChRs spontaneously. The potential of agrin to induce a signalling cascade leading to full clustering of AChRs is kept in check by SHP-2, since blocking SHP-2 leads to an increased agrin induction potential. This was however insufficient to bring the level of AChR clustering to the same level as in the control during the 16 h of agrin induction. SHP-2 activity is also required for the maintenance of stable AChR clusters following agrin withdrawal. SHP-2 thus seems to have three roles. Firstly it allows myotubes, overall, to bear high numbers of AChR clusters. Secondly, it limits agrin's potential to induce clusters. Finally, it stabilises clusters induced by agrin.

**Figure 3.6. Functional knockdown of SHP-2 in C2 myotubes reveals its role in AChR clustering, and in stability of these clusters upon agrin withdrawal.** (A) The effect of SHP-2 knockdown on spontaneous and agrin-induced AChR clusters, and on the stability of these clusters following agrin withdrawal was analysed. *pSUPER*-, and *pSUPER.shSHP2.2*-transfected C2 myotubes were either left untreated (No Agrin), treated with agrin for 16 h (Agrin), or treated with agrin (16 h) followed by withdrawal of agrin for 8 h (Agrin + Withdr.). AChRs were identified by rhodamine-α-BTX staining, cells were fixed in -20°C methanol and analysed by fluorescence microscopy. Scale bar, 40 μm. Positively-transfected myotubes expressing EGFP indicated that transfection efficiency at the myotube level approached 100%. (B) The number of AChR clusters per field was calculated (as in Figure 3.1B) and is shown (means ± SEM, $N = 30$ from four similar experiments; ***$p < 0.0001$; two-tailed unpaired *t* test). SHP-2 knockdown results in over 81% loss in spontaneous AChR clusters compared to control transfected myotubes. As a consequence, agrin induction appears to be insufficient to induce clustering to levels of control transfected myotubes, with 58% less AChR clusters in *pSUPER.shSHP2.2*-transfected myotubes. There are also significantly less agrin-induced AChR clusters upon gene-specific targeting of SHP-2 following agrin withdrawal. (C) Spontaneous AChR clusters are reduced by over 81% upon targeting of SHP-2 by shRNA (***$p < 0.0001$; two-tailed unpaired *t* test). (D) An analysis of the strength of agrin to induce AChR clusters was carried out by normalising the number of induced AChR clusters following incubation with agrin, to the level of spontaneous AChR clusters present before induction. The analysis shows that knocking down SHP-2 results in a highly significant 2.2-fold increase in the potential of agrin to induce AChR clusters (***$p < 0.0001$; two-tailed unpaired *t* test). (E) The number of AChR clusters in agrin-treated myotubes was set to 100%, both for untransfected as for transfected myotubes. Following agrin withdrawal, the absence of SHP-2 renders agrin-induced AChR clusters significantly less stable (over 27%) when compared to control transfected myotubes (*$p < 0.05$; two-tailed unpaired *t* test). These results indicate that SHP-2 plays important roles in controlling the ability of surface AChRs to cluster spontaneously and in negatively-controlling the potential of the agrin induction leading to clustering. They also show an important novel role for SHP-2 in maintaining the stability of AChR clusters following agrin withdrawal.


A
EGFP
R-BTX
EGFP
R-BTX
No Agrin
Agrin
Agrin + Withdr.
Transfection:
pSUPER
pSUPER.shSHP2.2
B
pSUPER
pSUPER.shSHP2.2
AChR clusters per field (400X)
***
no agrin
agrin
agrin + withdr.
C
AChR clusters per field (400X)
pSUPER vector
pSUPER.shSHP2.2
D
Agrin induction of AChR clusters (% of no agrin control)
E
AChR cluster stability (% of agrin-treated)
*

### *3.4.7 Elevated levels of SHP-2 in the absence of Src and Fyn*

We have observed similar destabilising effects on AChR clusters in wild-type myotubes upon PTP inhibition by pervanadate, and upon knockdown of SHP-2 by shRNA (Figures 3.1 and 3.6). This instability is also present in the absence of Src and Fyn in myotubes (Smith et al., 2001), or upon blocking SFKs with the inhibitor PP2, and is rescued completely by inhibition of phosphatase activity by pervanadate (Figure 3.2). These observations lead to the conclusion that it is the balance between PTK and PTP activity that regulates the stability of AChR clusters in myotubes. For this reason we sought to compare the levels of SHP-2 between *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes and wild-type myotubes. We therefore carried out Western blot analysis of myotube lysates, and found that SHP-2 levels are 3-fold higher in myotubes lacking *src* and *fyn* compared to wild-type myotubes (Figure 3.7). This finding further strengthens the hypothesis that it is the balance between Src and Fyn, and SHP-2, which controls AChR cluster stability. In *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes, elevated SHP-2 levels could be causing the instability of AChR clusters. Inhibiting these myotubes with pervanadate brings down the phosphatase activity, returning stability to the system, and allowing for more stable AChR clusters. On the other hand, in wild-type myotubes, levels of SHP-2 are normal. Thus, on phosphatase inhibition by pervanadate, the phosphatase activity is reduced below normal levels, tipping the balance towards increased PTK activity, and leading to a destabilisation of the system and consequently of AChR clusters.

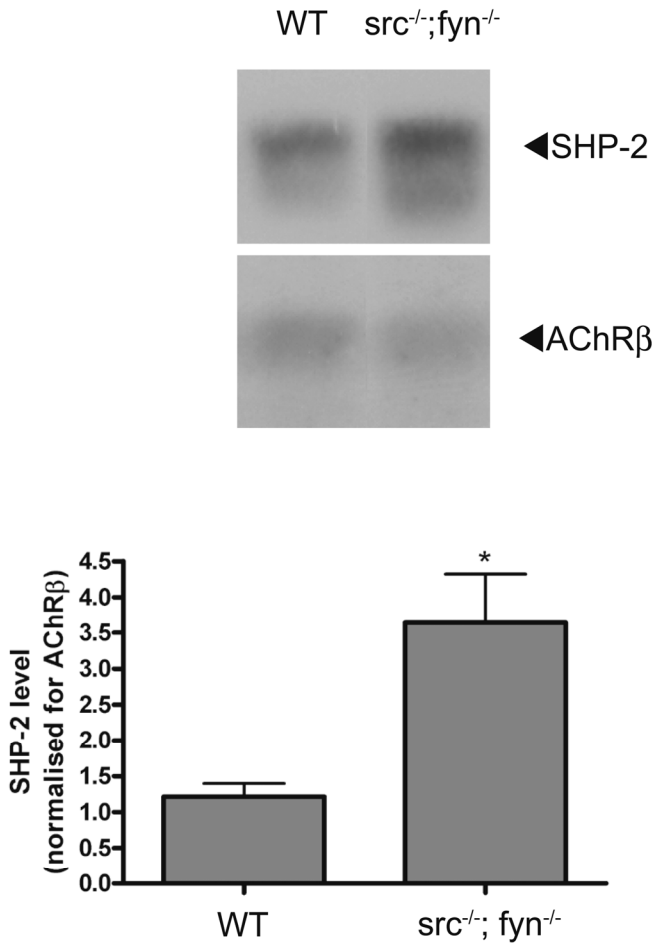



**Figure 3.7. Elevated levels of SHP-2 in the absence of Src and Fyn.** Western blot analysis of total SHP-2 protein levels in wild-type versus *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes reveal that in the absence of Src and Fyn there is a 3-fold increase in the expression of SHP-2 compared to wild-type. SHP-2 levels are shown for both wild-type and *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes, normalised for the level of AChR β subunit (no difference between the two cell lines) and loading equal protein quantities on the gel (*$p < 0.05$; two-tailed unpaired *t* test).

## 3.5 Discussion

This study reveals the requirement for a balance between PTP activity and SFK activity in the control of agrin-induced AChR cluster stability following the withdrawal of agrin in mouse myotube cultures. New insights into the roles played by PTPs during the spontaneous formation of AChR clusters and the formation of agrin-induced AChR clusters are also provided. We show that Src activity may be positively controlled by PTPs, and that this may partly explain the destabilisation of AChR clusters following inhibition of PTPs by pervanadate. The non-receptor PTP SHP-2 associates with MuSK increasingly upon MuSK activation by agrin. We knocked down SHP-2 by shRNA in myotubes and show that it is necessary for spontaneous clustering of AChRs. SHP-2 is required for stability of agrin-induced AChR clusters following the withdrawal of agrin in C2 myotube cultures. During agrin-induced MuSK signalling leading to AChR clustering, SHP-2 also acts to dampen the induction potential of agrin.

### *3.5.1 Phosphatase activity required during AChR clustering*

Disturbance of PTP activity by the potent inhibitor pervanadate in mouse myotubes leads to a decreased level of AChR clustering following stimulation by agrin. This occurs independently of modulation of MuSK phosphorylation. The inhibitory effect on AChR clustering may stem from strong phosphorylation of AChR β subunits by pervanadate. Such phosphorylation appears to link the receptor to the cytoskeleton locally (Wallace, 1995). Our data further suggest that the extent of disruption of PTPs governed by the preparation method of inhibitor, timing of inhibitor treatments, concentration of the inhibitor, and timing and concentration of agrin stimulation, could all influence the balance between PTP and PTK activity in myotubes. Under our experimental conditions, pre-incubating myotubes with 20 μM sodium pervanadate, followed by a 6-8 h induction by agrin (1 nM), leads to a significantly lower number of AChR clusters induced by agrin as compared to controls. This result is in line with

previous findings (Meier et al., 1995; Wallace, 1995), but differs from findings published recently by Madhavan et al. (2005), where, using 10 μM pervanadate added directly at the time of agrin addition, the authors found no change in the number of agrin-induced AChR clusters upon pervanadate treatments of C2 myotubes. Cluster size was however increased by pervanadate in the Madhavan et al. (2005) study, leading to sometimes long clusters of low intensity. One possible explanation of these contrasting observations are the different experimental conditions used in our study. We tested several concentrations of pervanadate (2-50 μM), however the outcome remained the same. We chose to use 20 μM since at this concentration pervanadate caused no observable toxicity to myotubes (judged from myotube morphology). Another explanation may be related to different threshold levels for counting AChR clusters. We noticed many low intensity clusters induced by pervanadate (sometimes occupying large areas; Figure 3.1). These could be similar to clusters counted by Madhavan et al. (2005), which we did not score in our study. Finally, the balance between PTPs and SFKs regulates AChR cluster stability (see below). Changes in this balance in different subclones of C2 myotubes, or in mouse versus chick myotubes, may lead to different effects of pervanadate on cluster induction.

Our results throw novel light onto the requirement of activity of one or more PTPs for AChR cluster stability, since pervanadate leads to a more rapid dispersal of agrin-induced clusters in myotube cultures following agrin removal.

### *3.5.2 Act to balance: PTPs and SFKs*

SFKs play important roles in phosphorylating the AChR and MuSK early on following agrin stimulation, and acting on AChR clusters early on during agrin induction in order to recruit a long-lasting stabilisation pathway to maintain clusters following agrin withdrawal (Mittaud et al., 2004). Our data reveal that a tight balance between the activities of SFKs and one or more PTPs is required following removal of agrin in maintaining stable AChR clusters: in both PP2-treated C2 and *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes, clusters are unstable but stabilised by pervanadate.

PTPs do not influence MuSK activity during this stabilisation pathway, since MuSK phosphorylation is unchanged by pervanadate treatments. This is different from Madhavan et al. (2005) who noticed elevated MuSK phosphorylation and AChR clustering after pervanadate treatment. The difference may be explained by differences in kinase to phosphatase balance in different subclones of C2 cells. In our studies, AChR β subunit phosphorylation does fall under the control of PTP activity since pervanadate enhances β phosphorylation both during agrin induction and after its withdrawal. This enhanced AChR β subunit phosphorylation, and the inhibition of agrin-induced AChR clustering in C2 cells are in agreement with Wallace (1995), who noticed the same effects. The hypothesis behind the observed effect is that phosphorylated AChRs are linked to the cytoskeleton locally, making them unavailable for agrin-driven clustering. In our experiments, the control of AChR β phosphorylation by pervanadate, however, does not seem to correlate with the stabilisation of AChR clusters in the absence of agrin. Elevated AChR phosphorylation therefore does not guarantee stable clusters. Our monitoring of Src activity by probing the effects of pervanadate on Src $pY_{418}$ during agrin induction and stabilisation pathways following agrin withdrawal show that the activity of SFKs is controlled, at least in part, by one or more PTPs, which by dephosphorylating the inhibitory $pY_{529}$ could control SFKs activity. This positive control on SFK activity by one or more PTPs could also take place indirectly, by controlling a kinase which would be responsible in phosphorylating the inhibitory $Y_{529}$ (Zhang et al., 2004). One possible candidate PTP that has been shown to both associate with and control Src activity is SHP-2 (Peng and Cartwright, 1995; Zhang et al., 2004). SHP-2 is a ubiquitously expressed cytoplasmic PTP having two *src*-homology 2 (SH2) domains and a phosphatase domain (Feng, 1999). SHP-2 is highly abundant in muscle fibres, and associates with the AChR *in vivo* via its SH2 domains (Mei and Si, 1995; Tanowitz et al., 1999). Peng and Cartwright (1995) showed that SHP-2 associated with Src, and *in vitro* studies revealed that Src phosphorylates SHP-2, and SHP-2 dephosphorylates Src at $Y_{529}$. Both events allow for positive regulation of Src activity by SHP-2. Further evidence by Walter et al. (1999) revealed that SHP-2 binds to the SH3 domain of Src, causing the activation of Src by a non-enzymatic mechanism. SHP-2 was also found to preferentially associate with membrane fractions of cells

overexpressing the phosphatase, and that this membrane association is important for SHP-2 activity (Walter et al., 1999). Taken together, these results raise the interesting possibility that SHP-2 could be recruited via its SH2 domains to phosphotyrosine residues of a membrane-associated protein such as AChR or MuSK, upon activation of this membrane protein. This would lead to SHP-2 activation, and possibility for it to activate Src either directly (Walter et al., 1999) or indirectly (Zhang et al., 2004). Src activity, in turn, could be controlled by SHP-2 or other phosphatases, revealing complex mechanisms underlying the net balance between kinases and phosphatases. From our data we can conclude that PTPs act to balance SFK activity in a stabilisation pathway, leading to stable AChR clusters after the removal of agrin.

### *3.5.3 Agrin brings SHP-2 closer to MuSK*

We identify for the first time an association between SHP-2 and MuSK. We show that the MuSK-SHP-2 association occurs under basal conditions in mature C2 myotubes, possibly due to basal levels of autophosphorylation of MuSK leading to recruitment of SHP-2 to the muscle membrane. We also show colocalisation of MuSK and SHP-2 on plasma membranes of heterologous cells transfected with wild-type MuSK and SHP-2 constructs. There is a 2.3-fold increase in MuSK-SHP-2 association upon agrin-induced phosphorylation of MuSK in myotubes. This is a similar-fold increase to SHP-2 association with tyrosine phosphorylated DOK1, a member of insulin receptor substrate protein family that binds β3. Association with DOK1 also occurs under basal conditions, and upon stimulation of DOK1 by insulin-like growth factor I (IGI-I), SHP-2-DOK1 association increases 2.7-fold. This association is important for DOK1 to present SHP-2 to downstream SHPS-1 (Ling et al., 2005; Ling et al., 2003). In a similar fashion, SHP-2 associates, via its SH2 domains, to other RTK including the platelet-derived growth factor (PDGF) and epidermal growth factor receptors and IRS-1 (Case et al., 1994). These studies provide examples which allow us to speculate that SHP-2 recruitment to tyrosine phosphorylated MuSK allows for proper SHP-2 localisation at the muscle membrane, and at sites where its phosphatase activity would be required. One possible downstream target of recruited SHP-2 is Src, which, as we have previously shown, is involved in MuSK and AChR β

phosphorylation (Mittaud et al., 2004), and SHP-2 is known to both interact (Walter et al., 1999) and positively regulate Src activity (Peng and Cartwright, 1995; Walter et al., 1999; Zhang et al., 2004).

### *3.5.4 Effective and efficient knockdown of endogenous SHP-2 in myotubes*

We achieved a highly efficient transfection of C2 myotubes using Fugene6, transfecting cells at the myoblast stage. Transfection efficiencies as high as 40-50% at the myoblast level led to 90-100% transfection efficiency at the myotube level following myoblast fusion, judged by the expression of EGFP co-expressed from the shRNA vector *pSUPER.gfp*. Hence we knocked down endogenous SHP-2 very efficiently using the *pSUPER.gfp* (Brummelkamp et al., 2002), following selection of the best candidate short-hairpin sequence adapted from three published candidates. The generation of shRNAs by *pSUPER.gfp* is driven by the polymerase-III H1-RNA (H1) gene promoter, which provides transfected cells with an efficient supply of intracellularly-produced small-interfering RNA (siRNA) duplexes (Brummelkamp et al., 2002; Tuschl, 2002). The processing of hairpin RNAs requires Dicer RNase III (Paddison et al., 2002), and allows for a stable supply of siRNA duplexes for an efficient gene knockdown. The knockdown of endogenous SHP-2 did not interfere with myoblast differentiation, in spite of the known requirement for SHP-2 during myogenesis (Kontaridis et al., 2004; Kontaridis et al., 2002). One reason for this may be that the intracellular pool of siRNAs, derived from the expression of the shRNA vector, did not reach a high enough level before myogenesis took place to cause a disruption of the differentiation process, but rather peaked later, following myotube maturation. The known role of SHP-2 in myogenesis illustrates the importance of verifying the normal fusion and differentiation of shRNA vector-transfected cells. Judging by the morphology of the myotubes under the microscope, they were normal and unaffected by the transfections. Furthermore, SHP-2 knockdown did not interfere with the expression of MuSK, Src, rapsyn, or β-dystroglycan. AChR β subunit expression was slightly affected since lower protein levels were detected by Western

blotting. We however saw no significant change on the number of functional AChRs inserted into the plasma membrane, as judged by our radioligand binding assays.

### *3.5.5 SHP-2 is required for clustering and stability of AChRs*

We targeted SHP-2 in C2 myotubes by shRNA, and investigated how the process of AChR cluster formation occurred both spontaneously and induced by agrin. We also investigated the stability of agrin-induced AChR clusters after agrin withdrawal. Myotubes lacking SHP-2 formed little or no spontaneous AChR clusters. The agrin induction potential was increased in the absence of SHP-2, but the level of AChR clustering reached was still not up to control levels after 16 h of agrin treatment. SHP-2 maintains agrin-induced AChR clusters stable following the withdrawal of agrin from myotube cultures. Thus, our results reveal multiple roles of SHP-2 during the clustering and maintenance of AChRs at the synapse.

AChRs cluster spontaneously in embryonic mouse muscle fibres even before the arrival of the motor neurone in a process termed pre-patterning (Lin et al., 2001; Yang et al., 2001). This pre-patterning occurs due the auto-phosphorylation of MuSK (Valenzuela et al., 1995) which is an essential event for proper AChR clustering *in vivo* (Lin et al., 2001; Yang et al., 2001). Intracellular domains of MuSK include a tyrosine kinase domain, and PTB and PDZ domain binding sequence motifs (Valenzuela et al., 1995). Herbst et al. (2002; 2000) showed that the tyrosine kinase domain and a juxtamembrane region tyrosine on MuSK are essential for downstream signalling events leading to clustering of AChRs. MuSK auto-phosphorylation at its intracellular tyrosine residues could easily lead to recruitment of SH2 domain containing proteins, such as SHP-2. The fact that we see a basal level of MuSK-SHP-2 association even in the absence of agrin is indicative of a possible mechanism for SHP-2 recruitment. The disruption of spontaneous AChR clusters caused by SHP-2 knockdown implies the requirement of SHP-2 for this early process to take place. AChRs are thought to form clusters as a result of the “diffusion-trap” principle in which freely moving receptors in the plasma membrane become trapped when reaching synaptic sites (Edwards and Frisch, 1976; Poo, 1985). One possible

"trap" could be the linking of AChRs to the actin cytoskeleton, shown by several groups to be important for clustering (Bloch, 1986; Connolly, 1984; Peng and Phelan, 1984). Strong AChR phosphorylation at β and δ subunits tightly links the receptor to the actin cytoskeleton (Mittaud et al., 2001; Qu and Huganir, 1994; Wallace et al., 1991) while β subunit phosphorylation is essential for AChR clustering (Borges and Ferns, 2001). MuSK-associated SHP-2 could control AChR phosphorylation either directly, or else indirectly via a kinase such as a member of SFKs, since SHP-2 is known to control Src activity (Walter et al., 1999; Zhang et al., 2004). We showed that inhibiting PTPs with pervanadate caused an increase in AChR β subunit phosphorylation. The hyperphosphorylation of the AChR β subunit could lead to strengthening of its link to the actin cytoskeleton, immobilising the receptor and rendering less prone to lateral diffusion and thereby clustering. A similar effect could be achieved in the absence of SHP-2 in our *pSUPER.shSHP2.2* transfected myotubes, explaining why we see less spontaneously aggregating AChRs. In contrast to us, Madhavan et al. (2005) observed increased AChR clustering following SHP-2 knockdown. MuSK was also hyperphosphorylated after the knockdown. The reasons for the differences remain unclear to us, but may be related to differences in the overall kinase to phosphatase balance in different subclones of cells. Moreover, the role of SHP-2 in myogenesis (Kontaridis et al., 2004; Kontaridis et al., 2002) makes it necessary to ascertain proper myotube fusion and differentiation following the knockdown procedure. While we deliver appropriate control experiments, Madhavan et al. (2005) did not address this point. Therefore, differences in the differentiation status of the myotubes may contribute to the variations in the experimental outcome.

Agrin, upon stimulation of MuSK, leads to downstream signalling in the muscle which results in the clustering of AChRs. This process requires rapsyn (Gautam et al., 1995), and several other players, as recently reviewed by Strochlic et al. (2005). We show here, using SHP-2 shRNA, that the agrin induction potential leading to AChR clustering is enhanced. This result is in line with the recent work by Madhavan et al. (2005) showing a similar-fold increase in the agrin induction upon SHP-2 siRNA. This shows that SHP-2 is a negative regulator of agrin signalling. We find that the result of increased agrin signalling upon SHP-2 targeting is still insufficient to cluster AChRs to control levels following 16 h of agrin induction. This may be due to

increased AChR phosphorylation and hence linkage to the cytoskeleton, even before the stimulation of agrin, as mentioned above, and hence reduction in surface mobility of AChRs. The clusters formed in the SHP-2-deficient myotubes could also originate in part from insertion of newly synthesised AChRs from intracellular pools, which take at least 2-3 h to reach the surface following biosynthesis (Devreotes et al., 1977; Gelman and Prives, 1996; Prives et al., 1976), and would require an even longer period of time to reach high enough levels at the surface to form sizeable clusters. Clustering of surface AChR takes 2-4 h, and peaks at 6 h following agrin induction, and requires that AChRs be phosphorylated and cytoskeletally linked prior to clustering taking place (Ferns et al., 1996; Moransard et al., 2003).

Much is known about pathways leading to clustering of AChRs, but little has been revealed about mechanisms which stabilise AChR clusters postnatally at the mature NMJ. Seronegative myasthaenia gravis patients producing autoantibodies against MuSK have decreased stability of their NMJ, showing the importance of MuSK for proper maintenance of the mature NMJ (Hesser et al., 2006; Hoch et al., 2001). Components of the postsynaptic utrophin-complex, including utrophin, dystrophin, α-dystrobrevin and dystroglycan, are also mediators of synaptic stability, but are not essential during initial NMJ formation (Grady et al., 1997b; Grady et al., 2000; Jacobson et al., 2001). SFKs are also important during AChR cluster stability. In *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes, removal of agrin or laminin induces rapid AChR cluster disassembly (Marangi et al., 2002; Smith et al., 2001). SFKs hold together aggregates of many postsynaptic proteins, which fall apart along with the disintegration of AChR clusters in cultured myotubes; and SFKs help maintain *in vivo* nAChR phosphorylation, cytoskeletal interaction and binding to rapsyn (Sadasivam et al., 2005). Using our cell culture-based model for studying stability of agrin-induced AChR clusters, together with shRNA targeting SHP-2 in C2 myotubes, we show that SHP-2 is required to maintain stable AChR clusters. Our results have also shown that PTP activity may control Src activity in myotube cultures. Thus, SHP-2 may be one more link in the activation of SFK activity leading to maintenance of postsynaptic stability. The knockdown of SHP-2 in our C2 cultures could be causing lack of, or deregulation of SFK activity, thereby leading to instability of AChR clusters upon agrin removal. This disruption in SFK activity would also parallel the observation of

unstable clusters upon agrin withdrawal seen in our PP2-treated wild-type cultures and our *src*$^{-/-}$;*fyn*$^{-/-}$ myotube cultures.

### *3.5.6 Imbalance between SFKs and SHP-2 destabilise AChR clusters*

We show that in myotubes lacking Src and Fyn, SHP-2 levels are increased. This suggests that either Src, or Fyn, or both, are important in regulating SHP-2 expression. The resulting increase in SHP-2 levels could lead to increased overall phosphatase activity in myotubes, resulting in a destabilisation of the kinase-phosphatase balance required for proper maintenance of intracellular signalling pathways (Hunter, 1995). This finding could also explain why in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes, pervanadate inhibition of PTPs led to increased AChR cluster stability following agrin withdrawal. Pervanadate could be acting to bring down the increased phosphatase activity in these myotubes caused by the increased SHP-2 levels, and thereby re-establishing the kinase-phosphatase equilibrium required for stable AChR clusters. In concordance with this, PP2 treatment (leading to reduced SFK activity) destabilised AChR clusters, and pervanadate treatment restored their stability. All these data illustrate the importance of SFK to phosphatase balance in the AChR stabilisation pathway.

### *3.5.7 Dynamic equilibrium and stability*

In this study we reveal the tight link between tyrosine kinase and tyrosine phosphatase signalling, and the importance of dynamic equilibrium to exist between the two for stability of AChR clusters, important for mature and fully-functional neuromuscular synapses (Figure 3.8). We show that the interplay between the activities of non-receptor PTP SHP-2 and the SFKs, and how disturbing their balance leads to instability of agrin-induced AChR clusters after agrin withdrawal. We also provide evidence of how the presence or absence of one component of this equilibrium affects the other side of the balance, as happens in the change in expression levels of SHP-2 in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes. The dynamicity of this equilibrium allowed a balance to be re-established following inhibition of phosphatase activity by pervanadate, leading to

stabilisation of previously unstable AChR clusters. We finally provide evidence for a novel role of the phosphatase SHP-2 in the stability of AChR clusters at the neuromuscular synapse.

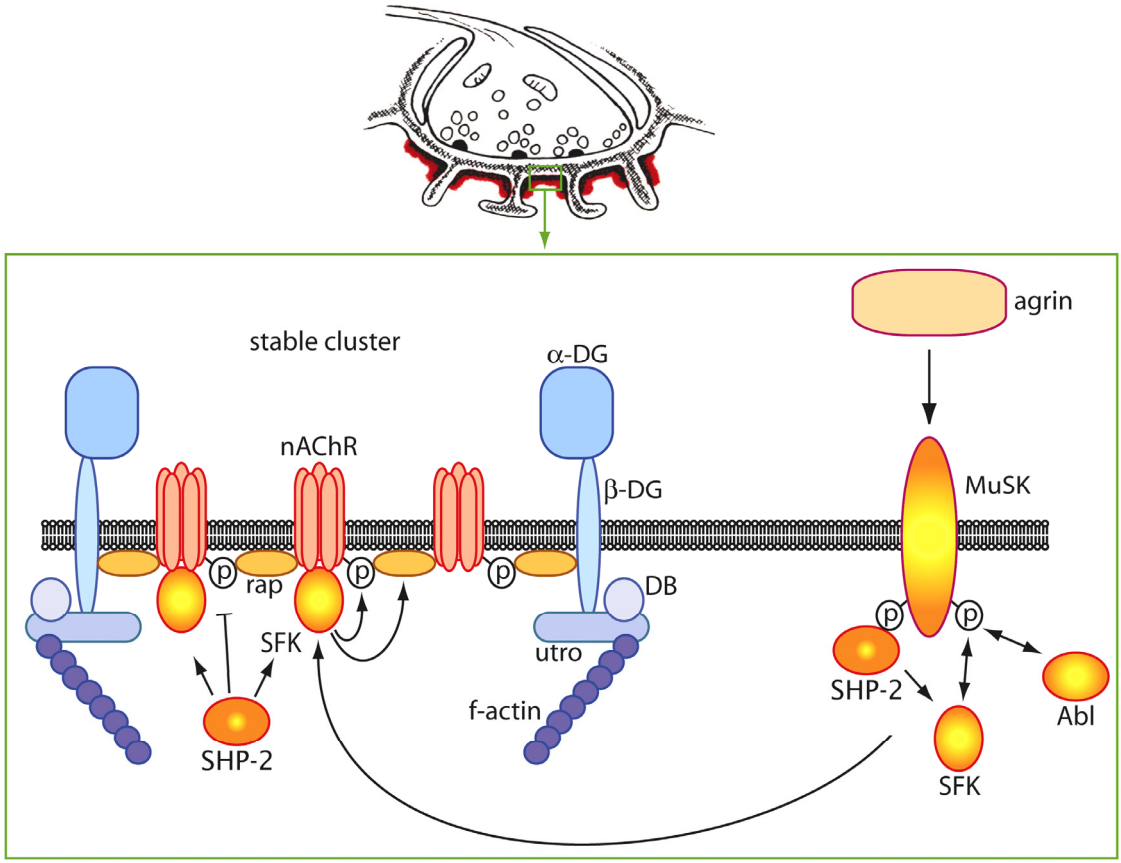



**Figure 3.8. A model of the roles of kinases and phosphatases controlling phosphorylation events and stable clustering of muscle AChRs in the postsynaptic membrane of the NMJ.** For details, refer to text.

# Chapter 4

## *Discussion*

The neuromuscular synapse has long served as a model for the study of the developmental processes occurring during synaptogenesis, ultimately leading to the formation of a functional connection exhibiting efficient synaptic transmission. The three strengths of the NMJ are its size, accessibility and simplicity (Sanes and Lichtman, 1999). The molecular events leading to developmental changes on pre- and postsynaptic densities of the NMJ also provide parallels for developmental events occurring in central synapses (e.g., for glutamate synapses see Husi et al., 2000). At the NMJ, the neurotransmitter acetylcholine (ACh), released from the presynaptic motor nerve, has to reach the apposed postsynaptic muscle membrane where it binds to the AChR, causing channel opening and local depolarisation. When a critical firing threshold is reached, voltage-gated sodium channels located in the troughs of postsynaptic folds open, and an action potential is initiated, leading to muscle contraction. This is only made possible by the fact that AChRs are clustered at very high densities (10-20,000 per $\mu m^2$) at the crests of postsynaptic junctional folds (Sanes and Lichtman, 1999). This clustering is a key aspect of postsynaptic development and of synaptogenesis as a whole. Agrin released from the presynaptic motor neurone triggers MuSK activation on the postsynaptic muscle and the formation of a primary synaptic scaffold, which appears to be a prerequisite for further downstream events to lead to AChR clustering (Glass et al., 1996). The signalling events taking place at the MuSK scaffold lead to the clustering of a rapsyn-AChR secondary scaffold at very high densities (Huh and Fuhrer, 2002). Evidence is emerging in recent years on the molecular signalling events taking place upon MuSK activation by agrin, the signals important for AChR clustering, and finally the signals which help to maintain a stable postsynaptic membrane with high densities of AChRs clustered there.

## 4.1 Summary of results

Key to agrin-induced signalling events are the activities of non-receptor tyrosine kinases and tyrosine phosphatases. Tyrosine phosphorylation is one of the most

important events in any signalling cascade, and interplay between PTKs and PTPs allows for a spatiotemporal control of signalling. It has been shown that apart from MuSK, the actions of other PTKs are necessary for both the clustering and stability of AChRs (Fuhrer et al., 1997; Wallace, 1994). SFKs and Abl kinases are both necessary for proper NMJ development (Finn et al., 2003; Mohamed et al., 2001; Sadasivam et al., 2005; Smith et al., 2001). They provide the kinase activity needed, following agrin stimulation, for driving the phosphorylation of MuSK and the AChR. I have shown, in **Chapter 2**, how these two PTK families allow for temporal regulation of the agrin signalling cascade. SFKs phosphorylate MuSK and the AChR early (after only 5 min) following agrin stimulation, whilst Abl kinases are necessary to phosphorylate MuSK and the AChR later (after 40 min of agrin). Their kinase activities therefore show temporal separation, but are also highly intertwined. The early signal generated by SFKs is not required for proper AChR clustering but is essential for stabilising AChR clusters both *in vitro* and *in vivo* (Sadasivam et al., 2005; Smith et al., 2001). On the other hand, the later signal provided by members of the Abl kinases is necessary for proper AChR clustering (Finn et al., 2003). My results point to a stabilisation pathway which is initiated very early on during agrin signalling by SFKs, and acts much later following clustering of AChRs in stabilising and maintaining these clusters (as judged from clusters remaining after the withdrawal of agrin in myotube cultures; **Chapter 2**). I also provide further evidence for the mode of action of agrin in orchestrating its signalling programme. A single brief agrin pulse, if it provides a sufficient level of MuSK phosphorylation, can drive a full clustering programme, independent of the continuous presence of agrin. This strongly implies that kinases and phosphatases downstream of MuSK keep the agrin signalling pathway activated, even in the absence of agrin.

The action of PTPs in regulating the phosphorylation events driven by agrin, and their role in the clustering of AChRs and the stability of these clusters, were studied in **Chapter 3**. I made use of the specific tyrosine phosphatase inhibitor pervanadate (PV), and showed that PTP activity is necessary for proper agrin-induced clustering and stability of AChRs. I extended this further and found that the dynamic equilibrium between SFK and PTP activities is actually the "phosphostat" ("phosphorylation thermostat") that regulates and governs the stability of agrin-

induced AChR clusters once agrin is removed from the cell culture medium. In **Chapter 3** I also identified a biochemical association between MuSK and the phosphatase SHP-2 in C2 myotubes, and colocalisation on membrane ruffles in transfected COS cells. The MuSK-SHP-2 association increases upon agrin-induced MuSK phosphorylation, allowing the possibility that MuSK is recruiting SHP-2 via association of a MuSK phosphotyrosine residue with either of the SH2 domains of SHP-2. This would allow for recruitment of SHP-2 to the MuSK primary scaffold, allowing for its phosphatase activity to be harnessed at the right place and at the right time. Using short-hairpin RNA (shRNA) knockdown of SHP-2, I provide evidence for the requirement for SHP-2 during all phases of AChR clustering. Spontaneous AChR clustering was reduced upon knockdown of SHP-2. Reduction of SHP-2 also led to reduced levels of agrin-induced clustering, in spite of the fact that the agrin induction potential was increased by blocking SHP-2 expression. Intriguingly, I observed a reduction in AChR cluster stability when SHP-2 was knocked down, showing the importance of SHP-2 in the maintenance of stable AChR clusters in the absence of agrin (following agrin withdrawal from cultures). I finally identified elevated levels of SHP-2 in *src*$^{-/-}$;*fyn*$^{-/-}$ compared to wild-type myotubes, which leads to the possibility that increased overall phosphatase activity together with the reduced kinase activity in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes, could lead to an imbalance in phosphorylation levels, causing instability of AChR clusters induced by agrin.

## 4.2 Tyrosine kinases

### *4.2.1 SFK activity: act early, profit later*

The actions of SFKs are important, firstly in phosphorylating MuSK and the AChR early on following agrin stimulation (within 5 min), and secondly in stabilising AChR clusters in the long term. In **Chapter 2** I showed how the activity of SFKs is required during the AChR clustering phase induced by agrin (8 h), for the later maintenance of stable AChR clusters once agrin has been removed and washed away from cells. Catalytically-active Src has also been observed to colocalise with AChR clusters after staining for Src $pY_{418}$ in *Xenopus* muscle cells (Madhavan and Peng, 2005). The

importance of SFK activity *in vivo* has been addressed recently by Sadasivam and colleagues (Sadasivam et al., 2005). They electroporated kinase-inactive Src constructs into soleus muscles of adult mice and observed the disassembly of existing NMJs characterised by fragmentation of AChR pretzels, disturbed topology of nerve terminal, AChRs and synaptic nuclei, and occasional nerve sprouting. Electroporation of kinase-overactive Src resulted in a similar but milder phenotype. The authors provided evidence for the possible mechanism of SFK action in postsynaptic stability, and found that in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes the agrin-induced interactions between AChRs and rapsyn, and AChRs and dystrobrevin were unstable after agrin removal. Rapsyn and proteins of the dystrophin/utrophin glycoprotein complex (D/UGC) were recruited normally into AChR clusters. However, upon withdrawal of agrin from *src*$^{-/-}$;*fyn*$^{-/-}$ cultures, the rapsyn and D/UGC clusters dissolved together with AChR clusters, showing the requirement of SFKs in maintaining complexes of AChRs and scaffolding proteins together to provide postsynaptic stability. Rapsyn levels were also elevated two-fold in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes, showing the importance of SFKs in regulating expression of rapsyn in myotubes (Sadasivam et al., 2005). It is interesting that, as shown in **Chapter 3**, the SHP-2 protein levels are elevated three-fold in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes (see Figure 3.7). Therefore SFKs appear to be important in regulating the expression of a number of diverse players at the NMJ, and in the absence of Src and Fyn, elevated levels of SHP-2 and rapsyn result. Elevated SHP-2 levels could possibly elevate the overall phosphatase activity in the myotubes. Whether the increased rapsyn levels are a consequence of these elevated SHP-2 (or phosphatase activity) levels, or whether they are independent of them, remains to be established. It is also interesting that upon inhibition of phosphatase activity by pervanadate, the instability of AChR clusters was rescued following agrin withdrawal from *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes (Chapter 3). It would be interesting to see whether the clusters of rapsyn and of D/UGC are also rescued through phosphatase inhibition, or whether their presence in clusters depends solely on Src and Fyn. This would provide insights into the role of phosphatases in stabilising the whole postsynaptic apparatus, including AChRs and other components.

### *4.2.2 Abl kinases: act later, profit sooner*

Abl kinases phosphorylate MuSK and the AChR β subunit only after 40 min of agrin stimulation (Finn et al., 2003; see Chapter 2). Abl kinases hence provide agrin-induced MuSK with the kinase activity signal amplification required for proper clustering of AChRs. The temporal separation of the kinase activities of Abl kinases from those of SFKs has interesting consequences. Finn et al. (2003) showed that the Abl kinases are actually required for proper AChR clustering. Abl kinases are also important regulators of the actin cytoskeleton via their phosphorylation of regulatory proteins (Lanier and Gertler, 2000; Pendergast, 2002) and through the catalysis of actin bundling (Van Etten et al., 1994; Wang et al., 2001). Their presence and activity at the postsynaptic apparatus could therefore provide mechanisms for AChR clustering by enhancing actin bundling, also a requirement for AChR clustering. SFKs, although phosphorylating MuSK and AChR β subunit earlier than Abl kinases (Chapter 2), are not required for AChR clustering but for stabilisation of clusters (Chapter 2; Sadasivam et al., 2005). Thus, SFKs and Abl kinases are functionally intertwined. Coordination of their activities in time and space has the consequence that the pathways for both cluster formation and stabilisation are initiated concomitantly, at the nascent postsynaptic specialisation. Synaptogenesis at the NMJ appears as a process in which different aspects are initiated at the same time but take effect in a defined temporal sequence.

## 4.3 Tyrosine phosphatases

Protein phosphorylation allows for rapid switching of the activities of cellular proteins from one state to another. A protein phosphorylation cycle involves distinct reactions in the forward and reverse directions, catalysed by PTKs and PTPs respectively (Figure 4.1). The level of tyrosine phosphorylation can therefore be regulated by changing the *rate* of the PTK and/or PTP reaction. This reaction therefore serves like a *switch* or *phosphostat* which allows for the switching from one state to another.

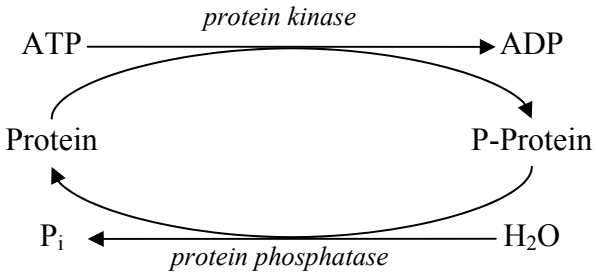



**Figure 4.1.** A protein phosphorylation cycle. Protein phosphorylation is an enzymatic reaction requiring the action of a PTK and the presence of ATP. Dephosphorylation of phosphorylated residues releases a phosphate group and requires the actions of PTPs. ATP, adenosine triphosphate; ADP, adenosine diphosphate; P, phosphotyrosine; $P_i$, inorganic phosphate; $H_2O$, water (adapted and modified from Hardie, 1995).

### *4.3.1 PTPs and agrin-induced AChR clustering*

The activity of PTPs during agrin-induced clustering of AChRs at the NMJ has been studied by the labs of Bruce Wallace and H.B. Peng (Dai and Peng, 1998; Madhavan et al., 2005; Meier et al., 1995; Wallace, 1995). In **Chapter 3** of this thesis, I have addressed the requirement of PTP activity during different stages of AChR clustering, namely spontaneous clustering, agrin-induced clustering, and for the stability of agrin-induced clusters once agrin is removed and washed away from cells. Similar to Wallace's study, I found that inhibiting phosphatases using pervanadate hindered agrin-induced AChR clustering and boosted phosphorylation of AChR β subunits (Wallace, 1995; Chapter 3). The hypothesis put forward by Wallace is that strong inhibition of phosphatases with pervanadate would increase the phosphorylation of AChRs and hence strengthen their link to the actin cytoskeleton. In fact, the mobility and extractability of AChRs in pervanadate-treated myotubes is decreased significantly (Meier et al., 1995). Borges and Ferns (2001) also provided evidence for the regulatory role of β subunit phosphorylation in AChR localisation, linkage of the AChR to the cytoskeleton and in progressively clustering the AChR at the nascent synapse. AChRs with mutated tyrosine residues in the β subunit were more detergent-extractable, and showed reduced clustering compared to wild-type in agrin-treated conditions. Cytoskeletal anchoring could occur via rapsyn-mediated linkage to the

cytoskeleton or indirectly by association with membrane proteins such as α- and β-dystroglycan (Borges and Ferns, 2001). From these and my data, one can conclude that PTP activity controls the phosphorylation of unclustered AChRs, an important factor for their link to the cytoskeleton. This control may occur via one of two mechanisms. The PTP may either be acting directly on the AChR by dephosphorylating tyrosine residues on it (Figure 4.2A). Alternatively one or more PTPs may indirectly inhibit the tyrosine phosphorylation of AChRs by negatively-regulating the kinase activity of a PTK responsible for its phosphorylation (Figure 4.2C).

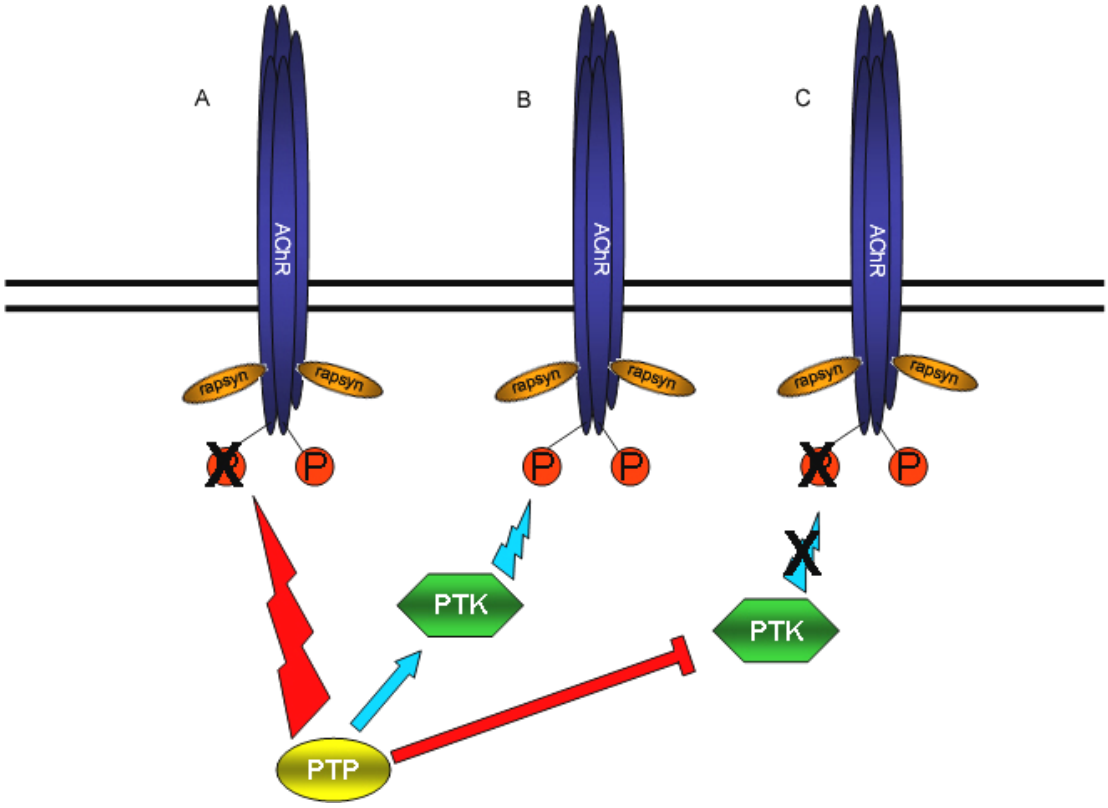



**Figure 4.2.** A scheme representing the possible mechanisms in which a PTP can control AChR phosphorylation. This could occur in one of three manners: (A) directly dephosphorylating a tyrosine residue on the AChR; (B) positively-regulating a PTK which in turn phosphorylates the AChR; (C) negatively-regulating a PTK which phosphorylates the AChR. Differences in the expression levels of PTPs versus PTKs, as may exist in different subclones of cultures myotubes or between different species (mouse vs. chicken vs. *Xenopus*), may lead to different effects on AChR clustering when PTPs are blocked by pervanadate. AChR, acetylcholine receptor; PTP, protein tyrosine phosphatase; PTK, protein tyrosine kinase; P, phosphotyrosine.

Therefore for AChR clustering to take place, sparsely-distributed AChR clusters, which are linked locally to the cytoskeleton, are helped to cluster by the action of PTPs which *dissolve* their links to the cytoskeleton, allowing them to move more freely in the mobile phases of the membrane, and to be more easily brought near sites of active clustering. H. B. Peng explains this phenomenon by using a model in which agrin-MuSK signalling triggers the activation of phosphatase activity downstream of MuSK, which diffuses to sites away from the MuSK scaffold, and allows pre-formed *hot spots* of AChRs to dissolve (Dai and Peng, 1998; Madhavan and Peng, 2003; Madhavan and Peng, 2005). Thus, when analysing roles of phosphatases in agrin-induced clustering, it is important to assess the effect of pervanadate on the phosphorylation status of the AChR (Figure 4.2). Madhavan et al. (2005) reported an increase in spontaneous clusters by pervanadate, and increased length (but unchanged numbers) of agrin-induced AChR clusters by pervanadate. They did not, however, analyse phosphorylation of AChR β subunit. Very likely, their phosphatase inhibition by pervanadate affected the phosphorylation status of the AChR in different ways than in our or Wallace's lab (Chapter 3; Wallace, 1995). This could easily explain the differences between the three studies (Chapter 3; Madhavan et al., 2005; Wallace, 1995).

### *4.3.2 PTPs and maintenance of AChR cluster stability*

In **Chapter 3** I investigated the role of PTP activity in the maintenance of AChR cluster stability. For this, I made use of a model in which AChRs are induced to cluster in myotubes by incubation with soluble agrin in the culture medium. Agrin is then removed from the medium, the cells washed, and incubated with fresh medium for a period of time, over which the stability of agrin-induced AChR clusters may be assessed. Using SFK inhibitors, and *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes, I show that SFK activity is required for the stability of AChR clusters (see Figure 3.2), in line with previous findings (Mittaud et al., 2004; Sadasivam et al., 2005; Smith et al., 2001). The role of SFKs in controlling AChR stability is discussed in more details above. Using the phosphatase inhibitor pervanadate I have also shown that in wild-type C2 myotubes, phosphatase activity is required for the stability of AChR clusters (see Figure 3.1 and

Figure 4.3). Conversely, phosphatase activity acts negatively on cluster stability in myotubes where SFK activity has been blocked or where Src and Fyn are absent (see Figure 3.2). Thus pervanadate stabilises clusters in these cells. For stable AChR clusters, a stable link is required between the AChRs and the cytoskeleton. This link is thought to be stabilised through the phosphorylation of the AChR (Borges and Ferns, 2001; Dai and Peng, 1998). In principle, phosphatases could affect stability of the postsynapse in three ways, which may act individually or in combination. AChR phosphorylation is one aspect in these mechanisms.

A. In a first scenario, phosphatases could be acting directly upon clustered AChRs, dephosphorylating them and loosening their link to the cytoskeleton, thereby somewhat reducing their stability. In this mechanism, PTP activity would be negatively regulating AChR cluster stability (Figure 4.3A).

B. A second possibility is that PTPs positively regulate a kinase which is responsible for phosphorylating AChRs. This PTP would therefore serve a positive role in stabilising AChR clusters (Figure 4.3B).

C. A third mechanism is one in which PTPs negatively regulate the action of a PTK which phosphorylates AChRs, thereby acting to reduce the phosphorylation of AChRs. This PTP would have a negative effect on AChR cluster stability (Figure 4.3C).

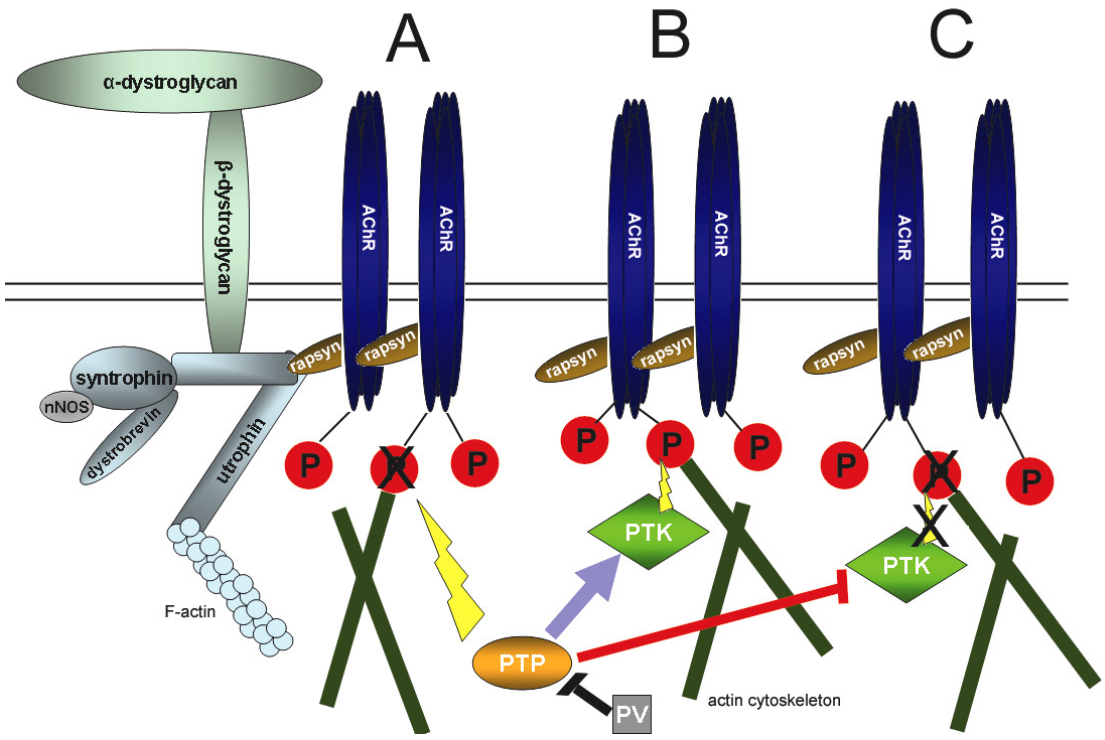



**Figure 4.3.** A schematic representation of three possible mechanisms of PTP action in controlling the AChR phosphorylation, either directly or indirectly, and thereby affecting their link to the cytoskeleton, and hence their stability. Combinations of several of these mechanisms is possible. Substrates other thatn the AChR are also involved (not shown). Overall, in C2 myotubes, blocking PTP activity with the inhibitor pervanadate (PV) creates instability of AChR clusters. AChR, acetylcholine receptor; PTP, protein tyrosine phosphatase; PTK, protein tyrosine kinase; P, phosphotyrosine; PV, sodium pervanadate.

Based on these three hypothetical mechanisms of PTP action in controlling AChR phosphorylation, link to the cytoskeleton and consequently AChR cluster stability, an interpretation of the results obtained in Chapter 3 can be made. One must however bear in mind that other subtrates are also involved besides the AChR. Inhibition of phosphatases using PV led to increased instability of AChR clusters in C2 myotube cultures following withdrawal of agrin. This means that PTP activity is having a stabilising effect on AChR clusters. Therefore, the possible mode of action of PTPs in this situation would be via mechanism B above, i.e. PTPs are probably positively-modulating the activity of a PTK which is responsible for AChR tyrosine phosphorylation that links the AChR more strongly to the actin cytoskeleton. In the absence of SFK activity (in PP2-treated C2 cells, or in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes) unstable AChR clusters are rescued by blocking the action of PTPs using PV. This means that

in such a situation, the action of PTPs is detrimental to AChR cluster stability, and PTPs could be acting via either mechanism A or C. Inhibiting PTPs would allow for reduced dephosphorylation of AChR clusters, or the permission of a PTK to phosphorylate the AChRs. The fact that the absence of SFK activity leads to unstable AChR clusters means that SFKs are possibly directly phosphorylating the AChR, and enhancing its link to the cytoskeleton. The absence of SFKs leads to a decreased overall kinase activity in the myotubes, and PTP activity could tip the phosphorylation balance, leading to more and more dephosphorylation of AChRs (through direct dephosphorylation). Pervanadate acts to re-establish AChR cluster stability by inhibiting the dephosphorylation of AChRs by PTPs, or by promoting PTK-driven phosphorylation of AChRs by inhibiting their negative-regulating PTP.

It must be added that the model in Figure 4.3 is a simplified one, focusing on AChR phosphorylation as the principal determinant of AChR stability through cytoskeletal link. Our preliminary analysis of AChR β subunit phosphorylation in pervanadate-treated C2 myotubes has shown an increase, yet clusters are unstable (Figure 3.3; Chapter 3). This implies that AChR phosphorylation is not the only determinant for cluster stabilisation. The phosphorylation status of other proteins, and combinations of the three possibilities in Figure 4.3, yield the picture of a complex array of proteins that influence cluster stability.

### *4.3.3 SHP-2: new player, multiple roles?*

Since protein tyrosine phosphorylation plays such important roles at the synapse, the search for effectors of these phosphorylation events has always been of great interest. Mei and Huganir (1991) performed a screen from *Torpedo* electric organ using $^{32}$P-labelled tyrosine phosphorylated AChR as a substrate, and purified and characterised a PTP that dephosphorylated tyrosine-phosphorylated AChR. Lin Mei later described unpublished results whereby using a polymerase chain reaction (PCR)-based strategy, eleven PTPs were identified from muscle. Among them was SHP-2, which was proposed to interact with the AChR through its SH2 domains (Mei and Si, 1995). SHP-2 was later found to colocalise with AChRs *in vivo* at the mouse NMJ,

and to negatively regulate the neuregulin signalling pathway thought to be involved in the expression of AChRs (Tanowitz et al., 1999).

The mammalian SHP-2 (Src homology-2 (SH2) domain-containing phosphatase 2) was cloned by a number of groups and thereby given several names: Syp, SH-PTP2, SH-PTP3, PTP1D, or PTP2C (Feng, 1999). Corkscrew (Csw), the *Drosophila* homologue, was identified in a genetic screen of chemically induced embryonic mutants (Perkins et al., 1992). Following its recruitment via its SH2 domains, SHP-2 propagates its downstream signalling and/or serves an adaptor function (Lechleider et al., 1993). SHP-2 recruitment serves both to localise it to the right location, as well as to activate it, allowing SHP-2 targets to be aptly activated in a coordinated and specific manner. SHP-2 may be tyrosine phosphorylated, leading to the recruitment of further downstream signalling complexes, in the case of Grb2-Sos in the epidermal growth factor (EGF)-stimulated EGF receptor (EGFR) leading to activation of the Ras signalling pathway (Li et al., 1994). In skeletal muscle, SHP-2 positively regulates myogenesis, by modulating the Rho family GTPase RhoA signalling pathway (Kontaridis et al., 2004). SHP-2 has also been shown to positively regulate Src activity (Zhang et al., 2004). Src is activated through the dephosphorylation of inhibitory $pY_{529}$, and phosphorylation of $Y_{418}$ (see Figure 2.1A). Zhang et al. (2004) showed how SHP-2 activates Src by decreasing Src $Y_{529}$ phosphorylation and increasing $Y_{418}$ phosphorylation. In SHP-2-deficient mouse fibroblasts, platelet-derived growth factor (PDGF), EGF and fibroblast growth factor (FGF) were unable to cause phosphorylation of Src $Y_{418}$, and thereby activate Src. SHP-2 overexpression in these mutant cells rescued the effect of the growth factors. In the SHP-2-deficient cells, the basal $pY_{529}$ phosphorylation was increased, and could not be decreased by growth factor stimulation. The mechanism by which SHP-2 regulates Src activity is summarised in Figure 4.4. SHP-2 dephosphorylates PAG, abolishing the Csk binding site on PAG, resulting in less Csk-mediated tyrosine phosphorylation of $Y_{529}$. This results in activation of Src by SHP-2 in a two-step process (Zhang et al., 2004).

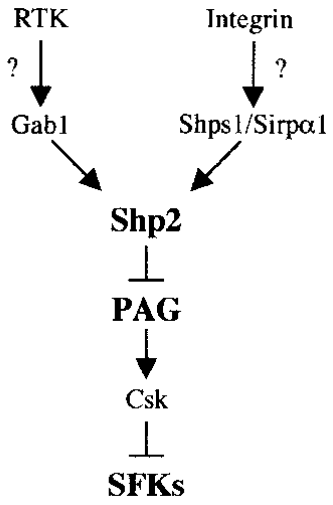



**Figure 4.4.** SHP-2 positively controls SFK activity. As a result of RTK or integrin stimulation, SHP-2 is recruited to the plasma membrane, most likely with the help of scaffolding adaptors such as Gab1, or Shps1/Sirpα1 glycoprotein. SHP-2 then dephosphorylates PAG, which regulates Csk recruitment, thereby promoting Src activation. Abbreviations: RTK, receptor tyrosine kinase; Gab1, GRB2-associated binding protein 1; Shps1, SH2-domain bearing protein tyrosine phosphatase (SHP) substrate-1; Sirpα1, Signal regulatory protein α1; PAG, phosphoprotein-associated with glycosphingolipid-enriched microdomains; Csk, C-terminal Src kinase (adapted and modified from Zhang et al., 2004).

In **Chapter 3**, I identified an association between MuSK and SHP-2 in mouse C2 myotubes (Figure 3.4). This association occurred under basal conditions, and was increased significantly by agrin-induced MuSK phosphorylation. Signalling is normally initiated when activated RTKs recruit downstream proteins, bearing SH2 or phosphotyrosine-binding (PTB) domains, to phosphorylated tyrosine residues contained within specific recognition sequences (Pawson, 1995). This association then often leads to phosphorylation of the recruited adapter protein and to further recruitment of components in a signal transduction cascade (van der Geer et al., 1994). This allows for the possibility that association between activated MuSK and SHP-2 occurs via an SH2 domain of SHP-2 and tyrosine phosphorylated residues on the C-terminus of MuSK. Agrin induces MuSK phosphorylation on six of nineteen of its tyrosine residues (Watty et al., 2000). MuSK phosphorylation by agrin could therefore enhance the recruitment of SH2 domain-containing signal transducers such as SHP-2. Src and Fyn have already been shown to interact with phosphorylated MuSK via their SH2 domains upon agrin stimulation (Mohamed et al., 2001). The recruitment of SHP-2 to a primary MuSK scaffold would allow for a SHP-2-induced

activation of SFK activity (perhaps through a mechanism similar to the one described above), which would lead to SFK recruitment to primary or secondary scaffolds at the NMJ. The activity of SFKs is consequently important both during early MuSK and AChR phosphorylation following agrin stimulation (Mittaud et al., 2004), as well as for the stabilisation of AChR clusters by tightening the link of receptors to the cytoskeleton (Mohamed et al., 2001; Sadasivam et al., 2005; Smith et al., 2001).

Bringing together the results of the studies outlined in Chapters 2 and 3 of this thesis, I propose the following mechanism for the regulation of the stability of AChR clusters by SFK and PTP and/or SHP-2 activity at the postsynaptic density of the NMJ. Agrin drives early stimulation of MuSK and AChRs via the activity of SFKs and Abl kinases. Upon phosphorylation of MuSK tyrosine residues, recruitment of SH2 domain-containing proteins, such as SFKs and SHP-2 occurs. SHP-2 recruitment to the primary scaffold allows for its catalytic activation. SHP-2, a positive regulator of SFK activity, drives the activation of SFKs and enhances their recruitment to both MuSK and AChRs, a process which is necessary for maintenance of MuSK and AChR phosphorylation. AChR phosphorylation at the site of clustering allows for a strong link to be created and maintained between the AChR and the actin cytoskeleton. Therefore both SHP-2 and SFKs act to increase AChR cluster stability by enhancing its phosphorylation and hence anchoring to the cytoskeleton (Figure 4.5).

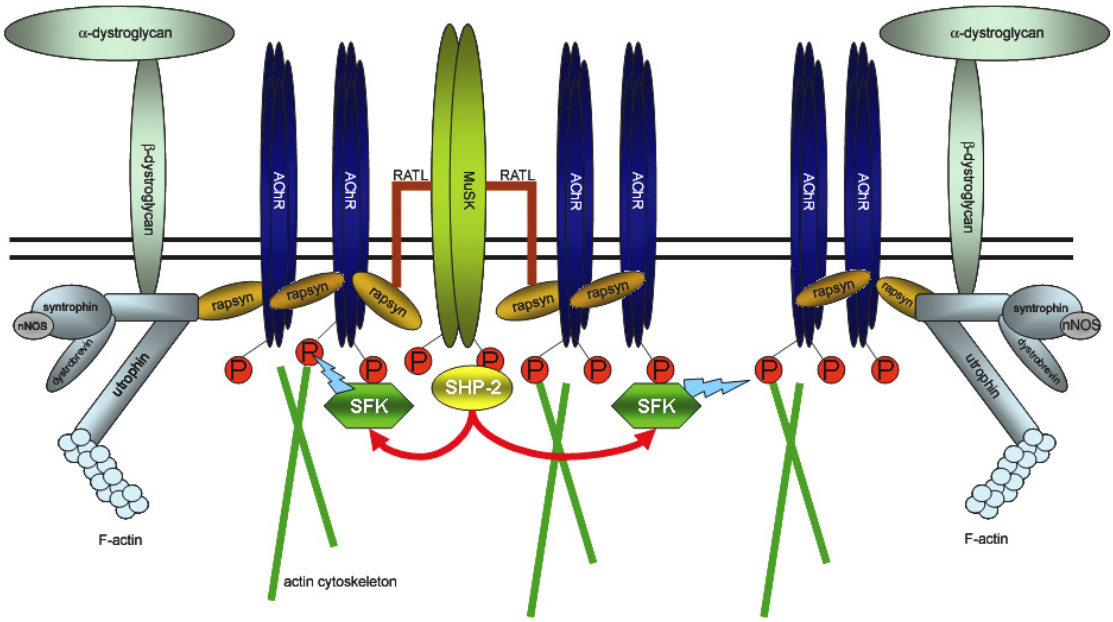



**Figure 4.5.** A schematic representation of the proposed mechanism involved in stabilising AChR clusters by the concerted action of SHP-2 and SFK recruitment, and the positive regulation of SFK activity by SHP-2. Further substrates besides the AChR are likely to also be involved. AChR, acetylcholine receptor; RATL, rapsyn-associated transmembrane linker; SFK, Src-family kinase; SHP-2, SH2 domain-containing protein tyrosine phosphatase-2; P, phosphotyrosine; nNOS, neuronal nitric oxide synthetase.

## 4.4 PTK vs. PTP activity: keeping the balance

Signalling cascades are initiated through the phosphorylation of tyrosine residues of RTKs. Proper signalling can only take place when these phosphorylation events are highly spatially- and temporally-regulated by means of a balance in the activity of PTKs and PTPs. Once the balance is lost, the resulting overactive kinase or phosphatase activity leads to changes in the signal cascade strength or outcome, with often undesired effects. This requirement for a fine PTK vs. PTP balance is clearly seen at the NMJ, where the balance between SFK activity and PTP activity is necessary for proper stability of AChR clusters. As seen in Chapter 3, the reduction in SFK activity created by the absence of Src and Fyn in knockout myotubes, or by treating C2 myotubes with the SFK inhibitor PP2, leads to highly unstable AChR clusters upon withdrawal of agrin from cell culture medium. A general reduction in the level of kinase activity in the myotubes, and therefore an imbalance in the PTK vs. PTP activity is created. However, upon treatment of myotubes with the PTP inhibitor

pervanadate, the balance is shifted back to a state of equilibrium, and the stability of AChR clusters is restored (see Figure 3.2). An interesting observation made in Chapter 3 was also that the protein levels of SHP-2 in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes were significantly elevated compared to wild-type cells. Consequently, in the absence of Src and Fyn in myotubes the overall PTP activity levels could be elevated. This imbalance causes instability, as seen by unstable AChR clusters, and the inhibition of PTPs in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes by pervanadate restores the stability to AChR clusters, perhaps through the restoration of the PTK vs. PTP equilibrium (see Figure 3.2). Upon knockdown of SHP-2 in C2 myotubes, instability of AChR clusters is also once again observed upon removal of agrin from the culture medium (see Figure 3.6). Yet again, the probable cause of such instability is an imbalance in the overall PTK vs. PTP activity.

In summary, our assays (Chapter 3) are the first to identify PTPs (as assessed by pervanadate treatments) as positive regulators of AChR cluster stability, and SHP-2 is a major PTP among them. SHP-2 may act by activating SFKs, possibly involving an indirect mechanism (Figure 4.4). In the absence of Src and Fyn or SFK activity, PTPs negatively regulate AChR cluster stability. This may be caused by overexpression of PTPs such as SHP-2 which dephosphorylate the AChR, reducing the cytoskeletal link of the receptor. Thus, PTPs such as SHP-2 can both increase AChR phosphorylation (by boosting SFK activity leading to receptor phosphorylation) and decrease AChR phosphorylation (by direct action on the receptor). This shows the importance of the balance between PTPs such as SHP-2 and SFKs in determining cluster stability.

With regards to spontaneous AChR clustering, our data show that SHP-2 positively regulates this process while other PTPs seem to negatively regulate it, such that overall inhibition by pervanadate produces no effect (Chapter 3).

For agrin-induced clustering, PTPs positively regulate this process while SHP-2 negatively regulates the agrin potential to form new clusters. Again, a balance between PTPs and SFKs is involved. This situation, and the differences to other results (Madhavan et al., 2005) shows that the PTP-PTK balance in spontaneous and agrin-induced clustering is complex. For example, SHP-2 knockdown increased clustering in Madhavan et al. (2005), but it was not certified that the knockdown only

affected levels of SHP-2 and not of other postsynaptic proteins. Differences in such control experiments, in species (chick and mouse), and in subclones of myotubes (possibly affecting PTK or PTP expression) could explain the different experimental outcome (Chapter 3; Madhavan et al., 2005; Wallace, 1995).

In any case, while more research seems necessary to provide insights into PTPs regulating cluster formation, the role of PTPs, SHP-2, and their interplay with SFKs in regulating cluster stability is clear from our studies.

## 4.5 PTKs and PTPs at CNS synapses

SFKs and PTPs do not only act in a synaptic context at the NMJ. Src has been implicated in proliferation and differentiation during the development of the CNS. It must also play additional functions after CNS development, since it is highly expressed in fully differentiated neurones in the adult CNS (Kalia et al., 2004). One of the major functions of Src is to upregulate the activity of *N*-methyl-D-aspartate (NMDA) receptors and other ion channels, therefore controlling critical aspects of synaptic plasticity in the brain, including learning and memory, and pain and epilepsy (Kalia et al., 2004). The balance between tyrosine phosphorylation and dephosphorylation controls NMDA receptor currents since inhibiting PTP activity leads to suppression of channel currents; conversely, increasing PTK activity (by the introduction of exogenous Src) leads to an enhancement of NMDA receptor currents (Wang and Salter, 1994; Wang et al., 1996). Therefore, NMDA receptors are regulated by opposing activities of PTKs (especially Src) and PTPs. STEP (striatal enriched tyrosine phosphatase) is a family of brain-specific non-receptor PTPs, which are present at the postsynaptic density (PSD) of glutamatergic synapses (Oyama et al., 1995). STEP forms part of the NMDA receptor complex, and therefore acts as an endogenous PTP that downregulates the function of NMDA receptors, by acting as a break to Src activity-induced upregulation (Pelkey et al., 2002). In the brain, Src activity is positively-modulated by the action of the receptor-like tyrosine phosphatase, PTPα, which selectively dephosphorylates $pY_{529}$, releasing Src from catalytic inhibition (Zheng et al., 1992). PTPα is also a component of the NMDA receptor complex, and it acts to upregulate Src activity, thereby enhancing NMDA

receptor activity, and hence also playing a role in induction of long-term potentiation (LTP; Lei et al., 2002).

A delicate balance between kinase and phosphatase activities is required for synaptic plasticity in hippocampal CA1 pyramidal cells (Colbran, 2004). Mechanisms of PTP regulation of metabotropic glutamate receptor (mGluR)-induced long-term depression (LTD) in CA1 synapses in the hippocampus have recently been demonstrated by Moult et al. (2006). They showed how PTP inhibition by orthovanadate or phenylarsine oxide (PAO) selectively blocked LTD induced by (*RS*)-3,5-dihydroxyphenylglycine (DHPG), a group I mGluR agonist. This mGluR-LTD is accompanied by a decrease in GluR2 AMPA receptor subunit phosphorylation which is also blocked by PTP inhibitors. Phosphatase inhibition also blocks the ability of DHPG to reduce the number of AMPA receptor clusters on the surface of dendrites. Therefore PTPs are activated postsynaptically, resulting in the tyrosine dephosphorylation and increased trafficking and removal of synaptic AMPA receptors during LTD (Moult et al., 2006).

The actions of PTKs and PTPs are also important in the formation of stable cell-cell adhesion by type I cadherins, which play essential roles during synapse formation and/or stability, and axon growth and guidance (Lilien and Balsamo, 2005). Type I cadherins associate via their cytoplasmic domain with β-catenin, and β-catenin with α-catenin. Binding of β-catenin is regulated through the tyrosine phosphorylation of three critical tyrosine residues, each targeted by one or more specific PTKs, including Abl kinases and SFKs: $Y_{142}$ by Fyn, Fer and cMet; $Y_{489}$ by Abl; and $Y_{654}$ by Src and EGFR. Phosphorylation at these tyrosine residues results in disassembly of the cadherin complex. Homeostatic mechanisms are therefore in place that limit the extent and duration or β-catenin phosphorylation, allowing rapid reversibility of the phosphorylation by the PTKs to take place. In fact, PTPs such as protein tyrosine phosphatase 1B (PTP1B) form part of the N-cadherin complex, and therefore provide rapid dephosphorylation of β-catenin. This PTK/PTP activity pair thereby acts to maintain a unique temporal, spatial, developmental and physiological balance in the cadherin-mediated adhesions (Lilien and Balsamo, 2005).

Taken together, PTPs and PTKs (such as Src) play important functional roles at CNS synapses, affecting receptor properties, trafficking, synaptic plasticity and cell adhesion. A role in AMPA receptor trafficking, in particular, offers interesting parallels to the roles of PTPs in AChR clustering at the NMJ.

## 4.6 Diseases of the NMJ

The development and maintenance of the postsynaptic density of the NMJ is crucial for proper functioning of the synapse during neuromuscular transmission. However, even if all goes well throughout the developmental programme, the NMJ is also subject to other hurdles which can affect its proper functioning. Neurotoxins from venomous plant or animal species, or auto-antibodies against synaptic components are two such problems. Auto-antibodies can lead to a number of neuromuscular diseases which attack components of the postsynapse, and which lead to inhibited neuromuscular transmission, causing muscle fatigue and weakness (Vincent, 2006).

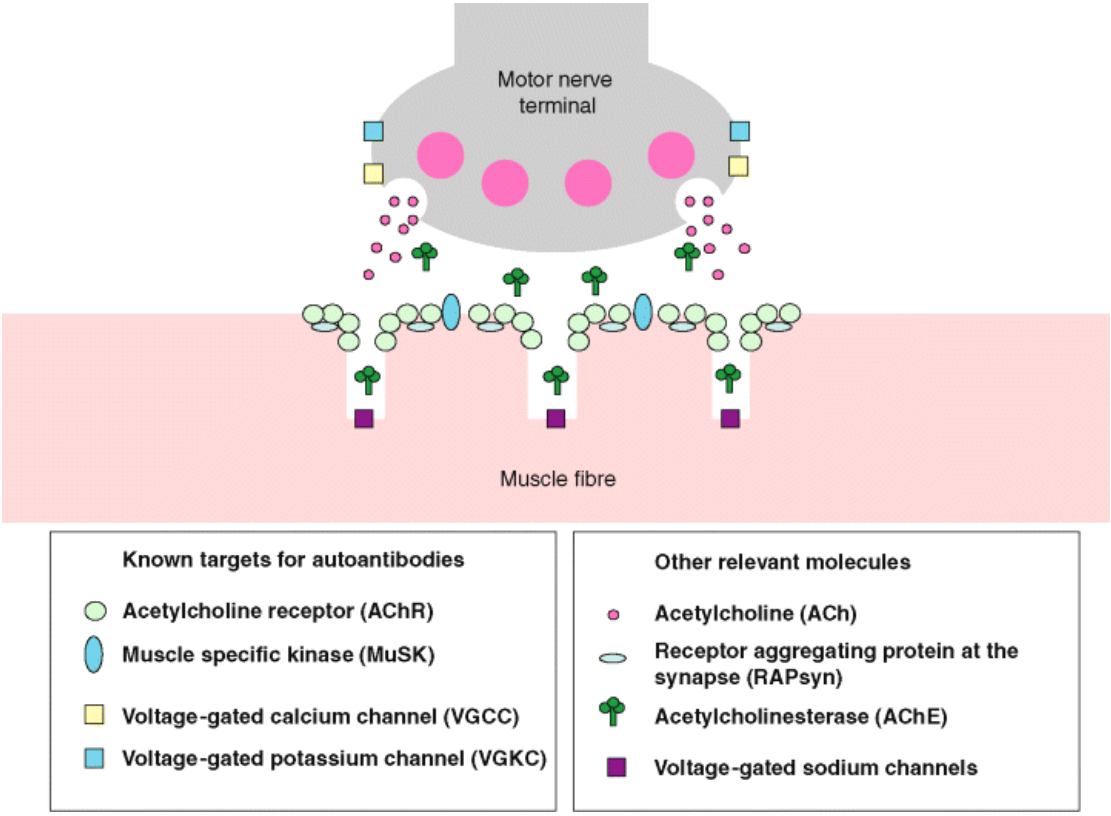



**Figure 4.6.** A schematic representation of sites of the NMJ, showing the main targets for auto-antibodies in myasthenic NMJ diseases (adapted from Vincent, 2006).

### *4.6.1 Myasthenia gravis: an autoimmune disorder of the NMJ*

Over 30 years ago, the discovery was made that a deficit in the levels of AChRs existed at the NMJs of patients suffering from myasthenia gravis (MG), an observation that was also confirmed in an animal model generated for the disease (Fambrough et al., 1973; Patrick and Lindstrom, 1973). MG is a disease characterised by muscular weakness and fatiguability caused by an antibody-mediated autoimmune attack, in 85% of cases directed against AChRs at NMJs (see Figure 4.6). Ocular and bulbar muscles are generally the most strongly affected (Drachman, 1994; Vincent, 2006). In 15% of patients exhibiting generalised MG symptoms however, no antibodies against AChRs are present, resulting in seronegative MG (Hoch et al., 2001). In 2001 Hoch et al. (2001) showed that in 70% of AChR-antibody-seronegative MG patients, serum auto-antibodies are produced against MuSK (Figure 4.6). This discovery opened the door for better diagnosis of the disease, with development of techniques to also measure the presence of MuSK antibodies in the

serum of patients suffering from MG. Apart from the AChR and MuSK, autoimmune diseases also arise due to auto-antibodies against voltage-gated calcium and potassium channels at the NMJ (Hoch et al., 2001).

What is the effect of producing auto-antibodies to the AChR or to MuSK? Direct blocking of the AChR or of MuSK may take place, causing effects on the ability of these receptors to signal or to respond to their ligands. Electron microscopy studies of NMJs from patients with MG revealed that morphological changes result, including flattening and simplification of postsynaptic folds (Drachman, 1994). Anti-AChR antibodies can also cause receptor internalisation, reducing amounts of AChRs at the surface (Fumagalli et al., 1982; Merlie et al., 1979; Stanley and Drachman, 1978). *In vitro*, anti-MuSK antibodies interfere with agrin-induced MuSK activation and AChR clustering (Hoch et al., 2001). The consequence of impaired AChR functionality or clustering at the surface are reduced amplitudes of endplate potentials, and postsynaptic folds distortion results in lowered numbers of voltage-gated sodium channels leading to increased thresholds for action potential generation (Vincent, 2006). The nerve terminal is however unaffected. Diagnosis to assess for MG involves an anti-AChE test, repetitive nerve stimulation, an assay for anti-AChR or anti-MuSK antibodies, and single-fibre electromyography (if necessary). Correct diagnosis of the disease is of utmost importance since patients who have been given a diagnosis of MG must usually undergo long-term medical or surgical treatment entailing considerable risks. Treatment can range from the administration of anticholinesterase drugs which enhance neuromuscular transmission, surgical removal of the thymus (thymectomy), immunosuppression, and short-term immunotherapies (such as plasma exchange and intravenous immune globulin; Drachman, 1994).

### *4.6.2 Other NMJ syndromes*

Postsynaptic aspects of the NMJ may not be affected solely by autoantibodies against targets such as AChRs or MuSK, but also by mutations in postsynaptic genes. Such mutations often lead to congential myasthenic syndrome (CMS), characterised by variable degrees of muscle fatigue (Byring et al., 2002; Engel et al., 2003).

The targets affected are once more AChR subunits and also MuSK (Chevessier et al., 2004; Engel et al., 1996; Ohno et al., 1995; Ohno et al., 1996). A relatively frequent cause are mutations in the gene for rapsyn, with many mutations having been identified (Ohno et al., 2002). AChR mutations affect receptor activity, but mutations in the genes for MuSK or rapsyn reduce the density of synaptic AChRs and their clustering.

Taken together, CMS and MG show that maintaining the postsynapse, with high-density AChR clusters, is essential for proper neuromuscular transmission. Proteins in the agrin signalling pathway – MuSK, rapsyn and AChRs – are key players in keeping our NMJs intact. Pathways of their regulation and clustering, thus, provide insights into mechanisms associated with neuromuscular diseases.

The integrity of the NMJ can also be affected at a presynaptic level. Lambert-Eaton myasthenic syndrome (LEMS) is a disorder affecting neuromuscular and autonomic synaptic transmission, and is caused by antibodies to voltage-gated calcium channels (VGCCs) at the motor nerve terminal (Figure 4.6). In half of all LEMS cases, the disease is associated with small-cell lung carcinoma (SCLC). Non-cancer cases are associated with a number of other autoimmune disorders. In LEMS, symptoms include muscle weakness in proximal muscles, especially leg muscles. The result of auto-antibodies to VGCCs is thought to involve cross-linking of the channels and internalisation (Evoli, 2006; Vincent, 2006). Neuromyotonia (NMT; Isaacs' syndrome) is a rare and heterogeneous syndrome of continuous motor unit activity of peripheral nerve origin that manifests as various combinations of muscle stiffness, cramps, twitching, weakness, and delayed muscle relaxation (Isaacs and Heffron, 1974). NMT can be either acquired or inherited. Acquired NMT is the auto-immune mediated form of the disease, in which voltage-gated potassium channels (VGKCs) on the presynaptic nerve terminal (Figure 4.6) can be the targets of antibody attack (Hart, 2000; Vincent, 2006). Morvan's *fibrillary chorea* or Morvan's syndrome is characterised by NMT, pain, hyperhydrosis, weight loss, severe insomnia and hallucinations. Like Isaacs' syndrome, it is also caused by auto-antibodies to VGKCs (Liguori et al., 2001).

## 4.7 A molecular understanding of the NMJ and parallels to other synapses

An elucidation of the molecular architecture of the NMJ is essential to fully appreciate the events surrounding its development and function. The NMJ offers a large and accessible model for the study of the synapse, the specialised point of contact between nerve and target cells. Understanding of molecular events happening both pre- and postsynaptically at the NMJ has also improved knowledge about CNS synapses. Many molecules important for postsynaptic development of the NMJ are also expressed in the PNS or CNS. One example is the D/UGC complex, which seems to assemble into functional scaffolding complexes in central synapses as it does in muscle (Imamura and Ozawa, 1998). The identification of molecules in central synapses serving parallel functions to molecules found at the NMJ will allow a better understanding of how CNS synapses assemble and function. At inhibitory $GABA_A$ synapses, the protein gephyrin, for example, appears to serve similar roles to rapsyn at the NMJ, in constituting a component of a protein complex with $GABA_A$ receptors and being required for clustering of certain of these receptors (Essrich et al., 1998; Kneussel et al., 1999). The SFKs are believed to serve varied functions at central synapses, not necessarily similar to the roles played at neuromuscular synapses. These include the regulation of glutamate synapses, where Src associates with NMDA receptors in a similar way as it does with muscle AChRs, NMDA receptors also being regulated by tyrosine phosphorylation (Yu et al., 1997). Src activates LTP in CA1 pyramidal cells of rat hippocampus through the potentiation of bound NMDA receptors (Lu et al., 1998). Cytoskeletal anchoring of CNS neurotransmitter receptors is also as important as it is for muscle AChRs at the NMJ. $GABA_A$ and glutamate receptors interact with the actin cytoskeleton by means of clustering molecules such as gephyrin and PSD-95 respectively, as well as through direct interaction (Garner et al., 2000; Kneussel and Betz, 2000). Members of the PSD-95 protein family (e.g. PSD-93) regulate the stability of interneuronal cholinergic synapses (and clusters of neuronal nicotinic AChRs) in rat superior central ganglion (Feng et al., 1998). In this ganglion, nicotinic receptor clustering and synapse formation require agrin (Gingras et al., 2002) but are independent of rapsyn (Feng et al., 1998).

In the chick ciliary ganglion, clustering of neuronal nicotinic AChRs at cholinergic interneuronal synapses requires APC protein, which interacts with PSD-93 (Temburni et al., 2004). APC is also required for AChR clustering at the NMJ (Wang et al., 2003), illustrating similarities in AChR clustering between the NMJ and interneuronal synapses in the PNS.

These parallels between CNS synapses, cholinergic synapses in the PNS, and the NMJ illustrate the model character of the NMJ and the need to more systematically identify core postsynaptic components for the development of excitatory synapses.

Finally, knowledge of events taking place during NMJ development has also allowed for a better understanding of molecular events surrounding certain autoimmune and genetic disorders such as MG or CMS (described above), and could lead to the development of better therapeutic approaches for the treatment of these disorders.

## 4.8 Perspectives

The NMJ is an old model of the synapse and of synaptogenesis. Poison arrows and their effect on muscular contraction led Claude Bernard, in 1856, to investigate further this specialised contact between the motor nerve and the muscle. Huge leaps have been made since then in the understanding of the events leading to the formation and stabilisation of this junction, and in the way it functions. More knowledge leads to old theories and models often becoming updated, or new models developed, eventually leading to their replacement by new paradigms. Big developments in the field often occur in parallel with technological developments, or the use of new tools. The age of molecular biology has been overtaken by that of genomics and proteomics, and the development of new imaging techniques, which allow for an *in vivo* glimpse at the dynamicity of the synapse during and after development. The importance of the actions of tyrosine kinase and tyrosine phosphatases in signalling cascades involved in the formation and stabilisation of the synapse have been outlined in this thesis. More knowledge will undoubtedly be acquired through more in depth analysis of the functions of individual molecules, with the use of upcoming techniques such as RNAi. The ability to use this tool *in vivo* in a variety of model organisms, coupled

with the possibility of switching a knockdown effect on or off at just the desired time point or desired place (such as with the use of inducible shRNA vectors, expressed conditionally in tissue-specific regions of an organism), will be a powerful tool capable of delivering new and precise details of the roles played by specific PTKs or PTPs in NMJ development. The conditional knockdown of proteins in a spatiotemporally controlled manner will also help to circumvent problems arising from early lethality of complete knockouts, such as is the case for SHP-2, in which knockout mice die during early gastrulation (Saxton and Pawson, 1999; Yang et al., 2006). Other innovative techniques, such as *in vivo* electroporation of constructs directly at the neuromuscular synapses, will continue to provide answers regarding the roles of specific proteins at the synapse (Kong et al., 2004). The next steps in fully elucidating the role of this fine balance between the activities of PTKs and PTPs during NMJ development would therefore be to analyse the effects of disturbing the function of such molecules using such *in vivo* techniques. This will allow novel paradigms to be developed that reflect more precisely the roles played by such proteins during the development of the NMJ.

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# CURRICULUM VITAE

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Mittaud, P.[*], Camilleri, A. A.[*], Willmann, R., Erb-Vögtli, S., Burden, S. J., and Fuhrer, C. (2004). A single pulse of agrin triggers a pathway that acts to cluster acetylcholine receptors. Mol Cell Biol *24*, 7841-7854. [*] *These authors contributed equally*.

Camilleri, A. A., Gesemann M., and Fuhrer, C. (2006). A balance between tyrosine phosphatases and Src-family kinases stabilises clusters of acetylcholine receptors. *Manuscript in preparation*.

**Posters/Presentations at Conferences:**

| | |
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| 18 January 2003 | Swiss Society for Neuroscience/Swiss Society of Psychiatry and Psychotherapy Joint Annual Meeting. Fribourg, Switzerland |
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| 17-19 February 2005 | Swiss Society for Neuroscience/Union of the Swiss Societies of Experimental Biology/Swiss Society of Biological Psychiatry Joint Annual Meeting. Zurich, Switzerland |
| 12-16 November 2005 | Society for Neuroscience (SfN) 35th Annual Meeting, Washington, DC, USA |

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Swiss Society for Cell Biology, Molecular Biology and Genetics

Swiss Society for Biochemistry

Society for Neuroscience (SfN)

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